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(54) Title: PROCESS FOR THE PRODUCTION OF POLYPEPTIDES IN MAMMALIAN CELL CULTURES

(57) Abstract: The invention provides a method for producing a polypeptide of interest which method comprises: providing a transformed host cell which comprises a nucleotide sequence which encodes a polypeptide of interest and which directs expression of the polypeptide of interest in the transformed host cell, or a hybridoma which comprises a nucleotide sequence encoding a polypeptide of interest; providing a culture medium suitable for mammalian cell culture; and culturing the hybridoma or transformed host cell in the culture medium under conditions that allow for expression of the polypeptide of interest, wherein the culture medium (i) comprises water, a buffer, an energy source, amino acids, a lipid source or precursor, a source of iron, non-ferrous metal ions, inorganic salts, vitamins and cofactors; and (ii) is free from each of a plant-derived or animal-derived peptone, a functional protein, and a full-length polypeptide. The invention further relates to a transformed host cell and to suitable low-cost media applicable in the method of the present invention.

PROCESS FOR THE PRODUCTION OF POLYPEPTIDES IN MAMMALIAN CELL
CULTURES

FIELD OF THE INVENTION

The present invention relates to a procedure for the cost-effective production of polypeptides of interest, particularly of therapeutic proteins, using mammalian cell lines.

5

BACKGROUND TO THE INVENTION

A cost-effective, commercial fermentation process for the production of polypeptides used as therapeutics such as, e.g., erythropoietin (Epo), human growth hormone (hgh), human monoclonal antibodies or monoclonal

10 human/mouse chimeric antibodies has to meet the following criteria:

(1) The cell culture medium should not contain any expensive compounds as for example serum or recombinant proteins.

(2) Due to a possible prion contamination, the medium should not contain any components of animal origin.

15 (3) The process should be scaleable and lead to a high final concentration of the recombinant protein of interest.

(4) The supernatant should contain a high amount of the polypeptides of interest displaying a high *in vivo* activity, so that the loss of less *in vivo* active isoforms during purification is diminished.

20

One technique for increasing the levels of expression is to transfect a vector containing the dihydrofolate reductase (dhfr) gene or a mutated form of dhfr (Banerjee D, Schweitzer I et al., Gene 139, (1994), 269-274) in addition to the human gene of interest, e.g. the human Epo gene, into dhfr deficient

25 CHO cells (Lee et al., 1999 (J. Biotech 69: 85-93) or other suitable mammalian cells such as baby hamster kidney (BHK) cells, human lymphoblastoid cells (Namalwa), human embryonic kidney (HEK-293) cells and HeLa cells. Addition of methotrexate (MTX) to the culture medium results in amplification of the dhfr gene and also the linked gene of interest.

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US Patent 5,441,868 describes in Example 10, page 28-29, a fermentation process consisting of three to four steps. In a first step the cells are seeded in a roller bottle (850cm^2) in 200 mL medium consisting of a 50:50 mixture of DMEM/Hams F12 and containing 5% fetal calf serum. After a 3-day period, when 5 the cells have grown to confluence, the medium is removed and replaced with a medium consisting of a 50:50 mixture of DMEM/Hams F12 and containing no fetal calf serum. The bottles are returned to the incubator for a period of 1-3 hours and the medium again removed and replaced with 100-mL of fresh serum-free medium. This step is used to reduce the concentration of contaminating 10 serum proteins. The roller bottles are then returned to the incubator for a seven day production period. After this time, the medium is collected and replaced with 100-mL of serum-free medium for a second production cycle. As an example of the practice of this production system a representative seven-day medium sample contains around $3,892 +/ 409 \text{ U/mL}$, corresponding to a 30 mg/L (at an 15 estimated specific activity of 130,000 U/mg).

This process, by using an expensive serum-containing medium, leads to a final concentration of 30 $\mu\text{g/mL}$ of erythropoietin (Epo) in the supernatant. Despite the fact that serum is strictly controlled, contamination with infectious agents and scPrions (most probably being responsible for transmitting CJD to humans) 20 cannot be excluded.

In US Patent 5,688,679, Example 5, the generation of a recombinant cell line is described leading to a final Epo concentration of 40 to 80 mg/L (at an estimated specific activity of 78,000 to 130,000 U/mg respectively). Among other animal derived components the cultivation media contain 20 % fetal calf 25 serum and 1 % bovine serum albumin. Such media suffer from the same prion and infectious agent-related problem mentioned above.

WO 96/35718 describes a process for the production of Erythropoietin which is free of animal derived components. However the medium contains expensive functional recombinant proteins like insulin and transferrin.

30 WO 99/28346 describes a fermentation process for the production of Erythropoietin using serum-reduced (1%) or serum-free medium. According to the description the final Epo concentration reached is at least 30 to 50 mg/L.

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However the medium still contains expensive functional recombinant proteins like insulin and transferrin.

Lee *et al.*, 1999 (J. Biotechnol. 69: 85-93) describes a process for the development of a cultivation media for a CHO cell line producing recombinant 5 human Erythropoietin using statistical design. By this approach a final Epo concentration of 25 μ g/mL was obtained. However, the media still contain expensive functional recombinant proteins like insulin and transferrin.

Accordingly, there is a need to develop a suitable culture medium and conditions for the production of polypeptides of interest, particularly recombinant 10 proteins, in the absence of expensive and undesirable animal-derived products and functional proteins such as insulin or transferrin.

SUMMARY OF THE INVENTION

The present invention provides a new fermentation protocol that uses a 15 cost-effective medium not containing serum or any functional full-length proteins. Furthermore, a number of parameters have been identified that may be used to optimize protein expression both in terms of yield and activity (due to a higher degree of sialylation), such as culturing cells in the absence of methotrexate. By using these optimized parameters, it has been possible to achieve a final protein 20 concentration of up to 600 mg/L, or even more, of the recombinant product (e.g. Epo) exhibiting a high degree of sialylation.

Accordingly, the present invention provides a method for producing a polypeptide of interest which method comprises providing a transformed host cell which comprises a nucleotide sequence which encodes the polypeptide of interest and which directs expression of the polypeptide of interest in the transformed host cell, or a hybridoma which comprises a nucleotide sequence encoding a polypeptide of interest; providing a culture medium suitable for mammalian cell culture; and culturing the hybridoma or transformed host cell in the culture medium under conditions that allow for expression of the polypeptide of interest, wherein the culture medium

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(i) comprises water, a buffer, an energy source, amino acids, a lipid source or precursor, a source of iron, non-ferrous metal ions, inorganic salts, vitamins and cofactors; and

(ii) is free from each of a plant-derived or animal-derived peptone, a functional protein, and a full-length polypeptide.

Further embodiments of the invention are defined in the dependent claims.

5 Advantageously, the medium is free from any component of animal origin.

Preferably the polypeptide of interest is selected from the group consisting of human growth hormone, human monoclonal antibodies, preferably of subclasses IgG, IgM, and IgA, and monoclonal human/mouse chimeric antibodies.

The hybridoma or transformed host cell is preferably derived from a mammalian

10 cell selected from the group consisting of rodent cells, particularly mouse hybridomas, human/mouse heterohybridomas, SP2-0 cells, NSO cells, baby hamster kidney cells, CHO cells, CHO dhfr⁻ cells, CHO-K1 cells, and recombinant cells obtained from any one of these cell lines..

In a preferred embodiment, the nucleotide sequence encoding a polypeptide 15 of interest is integrated into the genome of the host cell and is operably linked to a nucleotide sequence encoding dihydrofolate reductase, and the host cell is cultured in the absence of methotrexate.

It is preferred that at least one, preferably at least two, optionally three or more, polynucleotide vectors that contain an expression cassette that directs 20 expression of a polypeptide of interest, be introduced into a mammalian host cell, wherein at least one vector contains a gene encoding a selectable marker (such as dhfr or mutated dhfr) that is amplified in response to a selection agent and, optionally, the other or at least one other vector contains a different selectable marker (such as a gene conferring resistance to an antibiotic such as neomycin).

25 In one embodiment of the invention, the expression system comprises a polynucleotide vector which comprises a nucleotide sequence encoding a polypeptide of interest, and another nucleotide sequence encoding a selection marker such as dhfr or mutated dhfr, and still another nucleotide sequence encoding another selectable marker such as a resistance to an antibiotic.

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However, resistance to an antibiotic is not an indispensable requirement according to the present invention.

For the sake of cost reduction and the ease of handling, it is preferred that the nucleotide sequence encoding dhfr or mutated dhfr is provided using an expression vector which may be commercially available and which does not encode a polypeptide of interest. Accordingly, it is preferred that the polypeptide of interest is located on a separate expression vector which optionally may further contain a nucleotide sequence conferring resistance to an antibiotic. The expression system used according to the present invention therefore typically comprises at least two polynucleotide vectors, wherein one vector encodes the polypeptide of interest, optionally together with a resistance to an antibiotic, and the second vector encodes another selectable marker such as dhfr or mutated dhfr.

In yet another embodiment, the expression system comprises more than one vector encoding a polypeptide of interest and optionally a selectable marker such as resistance to an antibiotic, together with a vector that encodes another selectable marker such as dhfr or mutated dhfr. Typically, such expression systems comprising two or more vectors encoding polypeptides of interest, are applied in a method for the production of a recombinant antibody according to the present invention, wherein each vector encoding a polypeptide of interest encodes a part of the antibody, for instance a heavy or a light chain or another antibody fragment. Usually, the polynucleotide vectors encoding the polypeptides of interest are identical or essentially identical except for the sequences encoding the polypeptides of interest. They may also differ from each other with respect to the presence and/or nature of a sequence encoding an antibiotic resistance. On the other hand, the backbone of the polynucleotide vector that encodes the selection marker dhfr or mutated dhfr may be different from the backbone of the other vectors, i.e. from the vectors encoding the polypeptide(s) of interest.

Typically, the nucleotide sequence encoding resistance to an antibiotic, encodes resistance to G-418 or neomycin, i.e. it encodes neomycin phosphotransferase. As will be apparent to those skilled in the art, nucleotide

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sequences encoding resistance to different antibiotics, particularly of the neomycin group, may also be used.

Accordingly, in one aspect the present invention provides a method for producing a polypeptide of interest wherein a transformed host cell comprises

(a) a first polynucleotide vector comprising a first nucleotide sequence that encodes a polypeptide of interest, and, optionally, a second nucleotide sequence that encodes a selectable marker; and a second polynucleotide vector comprising a third nucleotide sequence that encodes a different selectable marker and that is free from a nucleotide sequence encoding a polypeptide of interest; or, alternatively,

(b) a polynucleotide vector comprising a first nucleotide sequence that encodes a polypeptide of interest, and, optionally, a second nucleotide sequence that encodes a selectable marker, and further a third nucleotide sequence that encodes a different selectable marker; and, optionally,

(c) at least one more polynucleotide vector comprising a nucleotide sequence encoding another polypeptide of interest and, optionally, a nucleotide sequence encoding a selectable marker;

the polynucleotide vectors being integrated into the genome of the transformed host cell.

Further embodiments of the invention are defined in the dependent claims.

5 Preferably, the nucleotide sequences encoding a polypeptide of interest and the nucleotide sequences encoding a selectable marker are operably linked to a strong promoter, typically a viral promoter such as an SV40 early promoter, CMV immediate early promoter or RSV-LTR promoter. Preferably, each of these nucleotide sequences is independently operably linked to such a strong promoter.

10 However, the use of respective polycistronic expression units will also be possible.

The method of the invention typically further comprises a step of recovering the polypeptide of interest from the culture. Where the polypeptide of interest is secreted into the culture medium, it will be recovered from the culture
15 medium.

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In another aspect, the present invention relates to a transformed host cell that is particularly suitable for the production of a recombinant polypeptide of interest according to the methods of the present invention and which transformed host cell comprises:

- (a) a first polynucleotide vector comprising a first nucleotide sequence that encodes a polypeptide of interest, and, optionally, a second nucleotide sequence that encodes a selectable marker; and a second polynucleotide vector comprising a third nucleotide sequence that encodes a different selectable marker and that is free from a nucleotide sequence encoding a polypeptide of interest; or, alternatively,
 - (b) a polynucleotide vector comprising a first nucleotide sequence that encodes a polypeptide of interest, and, optionally, a second nucleotide sequence that encodes a selectable marker, and further a third nucleotide sequence that encodes a different selectable marker; and, optionally,
 - (c) at least one more polynucleotide vector comprising a nucleotide sequence encoding another polypeptide of interest and, optionally, a nucleotide sequence encoding a selectable marker;
- the polynucleotide vectors being integrated into the genome of the transformed host cell.

Further embodiments of the invention are defined in the dependent claims.

The invention also relates to the use of a hybridoma or transformed host cell disclosed herein, in a method for producing a polypeptide of interest according to the present invention. The present invention also provides a composition comprising a polypeptide of interest produced by the method of the invention, wherein the polypeptide of interest is preferably selected from the group consisting of human growth hormone, human monoclonal antibodies, preferably of 5 subclasses IgG, IgM, and IgA, and monoclonal human/mouse chimeric antibodies. 10

In yet another aspect the present invention relates to an inexpensive, protein-free and serum-free cell culture medium, particularly to a cell culture medium suitable for culturing mammalian cells, characterized in that it

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(i) comprises water, a buffer, an energy source, amino acids, a lipid source or precursor, a source of iron, non-ferrous metal ions, inorganic salts, vitamins and cofactors; and

(ii) is free from each of a plant-derived or animal-derived peptone, a functional protein, and a full-length polypeptide.

Further embodiments of the invention are defined in the dependent claims.

In yet another aspect, the invention relates to a stunningly simple, yet

5 effective medium for freezing and/or maintaining eukaryotic, particularly mammalian cells. More particularly, the invention relates to a medium suitable for freezing and/or maintaining a mammalian cell, which medium consists of water, a buffer, preferably PBS, and at least one cryoprotective agent, preferably selected from the group consisting of dimethylsulfoxide (DMSO) and polyvinylpyrrolidone
10 (PVP).

Typically, DMSO and PVP are simultaneously present in the freezing and/or maintaining medium. Further embodiments of the invention are defined in the dependent claims.

In another aspect, the invention relates to the use of any medium disclosed
15 herein, in a method for producing a polypeptide of interest involving a hybridoma or transformed eukaryotic host cell expressing said polypeptide of interest.

Preferably, the polypeptide of interest is selected from the group consisting of human growth hormone, human monoclonal antibodies, preferably of subclasses IgG, IgM, and IgA, and monoclonal human/mouse chimeric antibodies.

It is also preferred that the hybridoma or transformed host cell is obtained from a mammalian cell selected from the group consisting of rodent cells cells, particularly mouse hybridomas, human/mouse heterohybridomas, SP2-O cells, NSO cells, baby hamster kidney cells, CHO cells, CHO dfr⁻ cells, CHO-K1 cells, and recombinant cells obtained from any one of these cell lines.

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DETAILED DESCRIPTION OF THE INVENTION

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art (e.g., in cell culture, molecular genetics, nucleic acid chemistry, hybridization techniques and biochemistry). Standard techniques are used for molecular, genetic and biochemical methods (see generally, Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.; Spier *et al.*, Encyclopedia of Cell Technology, Vol 1 & 2, 1st ed, (2000) John Wiley & Sons, Inc. and Ausubel *et al.*, Short Protocols in Molecular Biology (1999) 4th Ed, John Wiley & Sons, Inc. - and the full version entitled Current Protocols in Molecular Biology, which are incorporated herein by reference) and chemical methods.

Definitions

A eukaryotic host cell as referred to herein may be any cell of eukaryotic origin, whether a primary cell or an established, immortalized cell, which may be adapted for the expression of heterologous polypeptides. Thus, the term, in its broadest embodiment, includes primary cells in culture, tissues and organs in organ culture, tissue implants, whole organisms and established cell lines. In a preferred embodiment, the term refers to cells in culture derived from established cell lines, such as rodent cells, particularly mouse hybridomas, human/mouse heterohybridomas, SP2-0 cells, NSO cells, baby hamster kidney (BHK) cells, CHO cells, CHO dhfr⁻ cells, CHO-K1 cells COS, HEK-293, Namalwa and HeLa cells, and recombinant cells obtained from any one of these cell lines.

Expression, as referred to herein, is the production of a polypeptide product from a nucleic acid coding sequence, typically by transcription/translation of the sequence. Expressed polypeptides may be secreted from the host cell or may accumulate therein; preferably, expression according to the present invention includes secretion of the polypeptides from the cells. Expression levels may vary, but are advantageously high; about 1%, preferably 10%, 25% or 50% or even more of the total protein produced by the cell may be the expressed polypeptide of interest.

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Unless expressly stated otherwise, the term "hybridoma" as used herein, and particularly in the claims, refers to hybridomas that are adapted to contain a nucleotide sequence encoding a polypeptide of interest, wherein the polypeptide of interest is not a recombinant polypeptide. Hybridomas that are used as host 5 cells for transformation with one or more vectors encoding a polypeptide of interest and, optionally, one or more selectable markers, are generally encompassed by the term "transformed host cells".

Suitable polypeptides of interest include proteins of pharmaceutical interest, such as Epo, clotting factors such as factor VIII and factor IX, coagulating agents 10 such as hirudin, hormones, including insulin, human and animal growth hormones, follicle stimulating hormone, luteinizing hormone, other therapeutic proteins such as antibodies and other immunoglobulins, alpha-globin, beta-globin, gamma-globin, granulocyte macrophage-colony stimulating factor, tumor necrosis factor, interleukins, macrophage colony stimulating factor, granulocyte colony stimulating 15 factor, mast cell growth factor, tumor suppressor p53, retinoblastoma, interferons, melanoma associated antigen or B7 and any other proteins whose expression is desired, especially in large quantities.

A "polynucleotide vector" is a nucleic acid vector which may be used to deliver a nucleic acid coding sequence to the genome of a cell, such that it will 20 integrate therein. Integration into the host cell genome leads to stable transformation (as opposed to transient transformation commonly associated with episomal vector maintenance) of the host cell and may be referred to as "stable integration". Polynucleotide vectors, which are described in more detail below, may be plasmids, infectious agents (viruses, virus-like particles, viral nucleic acid), 25 cosmids, phagemids, transposons, packaged DNA or any other form of nucleic acid which is suitable for delivery to cells. Typically, plasmids are employed due to their ease of use, familiarity in the art and freedom from regulatory constraints required for infectious agents such as viruses. "Integration", or "stable integration", refers to the integration of at least part of the nucleic acid sequences 30 of the vector into the genome of the host cell or its maintenance in an artificial chromosomal body; at least the first and second/third nucleotide sequences are

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preferably integrated into the host cell genome. Advantageously, the entire sequence of the vector is integrated into the host cell genome.

"Transformation" refers herein to the introduction of genetic material into a cell such that the genetic material is expressed within the cell, which is said to be 5 "transformed". For the avoidance of doubt, "transformation" does not refer to the immortalization of cells by oncogenes or oncogenic viruses and the like.

"Amplification" refers to the increase in copy number of coding sequences in response to selective pressure. Thus, it is known that, in response to selection using MTX, heterologous dhfr genes are amplified and increase in copy number at 10 the locus in which they are integrated into the host cell genome. Sequences linked to the dhfr genes (i.e., sequences physically proximal thereto) are amplified together with the dhfr genes. This leads to increase in copy number of sequences of interest, and increased expression of gene products encoded thereby.

"Selection agents" are typically agents which place cells under stress or 15 other competitive disadvantage, unless a certain gene product is present in the cell which alleviates the effects of the selection agent. Thus, methotrexate is toxic to cells, but is neutralized by dihydrofolate reductase (DHFR), the gene product of the dhfr gene. Common amplifiable systems include dihydrofolate reductase (DHFR) – methotrexate (MTX), the bacterial xanthine-guanine 20 phosphoribosyl transferase enzyme (that mediates resistance to mycophenolic acid), and amplifying in the presence of a normal functioning gene (such as amplifying glutamine synthetase in the presence of methionine sulfoximine [MSX]). Such systems are amplifiable as such in that the nucleic acid is itself amplified in response to selection pressure applied by cognate agents.

25 Systems which are not usually regarded as amplifiable as such include, for example antibiotic resistance genes, which encode gene products which confer resistance to antibiotics such as ampicillin or G-418/neomycin.

The vectors according to the invention possess sequences which may be similar apart from the selectable markers and/or apart from the sequences 30 encoding the polypeptide(s) of interest. The first selectable marker is an amplifiable gene; the second need not be amplifiable. As used herein, "essentially the same" means that the sequences of the vectors encoding a polypeptide of

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interest, with the exception of the selectable marker sequences and/or the sequences for the polypeptide of interest, are identical or nearly so. In practice, two vectors encoding different polypeptides of interest or parts thereof are typically based on the same starting vector. Advantageously, the backbones of 5 the vectors, i.e. without the sequences of the genes of interest and without the marker genes and their respective control regions, may be more than 85% identical; advantageously, 90% identical; and preferably at least 95%, 98%, 99% or even completely identical. However, identity or essential identity of the vector backbones is not an indispensable requirement of the present invention.

10

Polynucleotide vectors

The term "operably linked" means that the components described are in a relationship permitting them to function in their intended manner. A regulatory sequence "operably linked" to a coding sequence is ligated in such a way that 15 expression of the coding sequence is achieved under conditions compatible with the control sequences.

Control sequences operably linked to sequences encoding the POI include promoters/enhancers and other expression regulation signals, including transcriptional terminators. These control sequences may be selected to be 20 compatible with the host cell for which the expression vector is designed to be used in. The term "promoter" is well-known in the art and encompasses nucleic acid regions ranging in size and complexity from minimal promoters to promoters including upstream elements and enhancers.

The promoter is typically selected from promoters which are functional in 25 mammalian cells. The promoter is typically derived from promoter sequences of viral or eukaryotic genes. For example, it may be a promoter derived from the genome of a cell in which expression is to occur. Viral promoters may advantageously be used since they generally direct high levels of transcription. Examples include the SV40 early promoter, and active fragments thereof, the 30 Moloney murine leukemia virus long terminal repeat (MMLV LTR) promoter, the Rous sarcoma virus (RSV) LTR promoter or the human cytomegalovirus (CMV) IE promoter.

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Since the replication of vectors is conveniently done in *E. coli*, an *E. coli* genetic marker and an *E. coli* origin of replication are advantageously included. These can be obtained from *E. coli* plasmids, such as pBR322, Bluescript[®] vector or a pUC plasmid, e.g. pUC18 or pUC19, which contain both *E. coli* replication 5 origin and *E. coli* genetic marker conferring resistance to antibiotics, such as ampicillin.

It may also be advantageous for the promoters to be inducible so that the levels of expression of the POI can be regulated during the life-time of the cell. Inducible means that the levels of expression obtained using the promoter can be 10 regulated.

The control sequences may be modified, for example by the addition of further transcriptional regulatory elements, for example enhancer sequences, to make the level of transcription directed by the control sequences more responsive to transcriptional modulators. Chimeric promoters may also be used comprising 15 sequence elements from two or more different promoters described above.

The first or at least one vector also comprises a second nucleotide sequence which is essentially an expression cassette which when integrated into the host cell genome is amplified when the host cell is contacted with a selection agent that causes amplification of the nucleotide sequence. Eukaryotic cells, in 20 particular mammalian cells are capable of amplifying certain genes in response to a selective pressure, typically an enzyme inhibitor, resulting in an increase in the numbers of copies of that gene, which in turn increases levels of expression of the gene. The additional gene copies may be maintained intrachromosomally, or in the form of extrachromosomal genetic material such as minute chromosomes 25 (for review see Genes VI, Lewin, 1997, Oxford University Press, Oxford U.K., pp975-978). A well-characterized example is the dihydrofolate reductase gene, including mutated forms thereof, which is amplified in response to methotrexate (MTX). Other examples include CAD gene (encodes a protein which is involved in the first three steps of UMP synthesis) which is amplified in response to inhibitors 30 of trans-carbamylase, and glutamine synthetase (see WO87/04462) which is amplified in response to methionine sulphoximine.

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This second expression cassette comprises the actually coding sequence of the gene that is capable of being amplified operably linked to a control sequence that is capable of providing for the expression of the coding sequence by the host cell. Suitable control sequences are described above. They may be the same or
5 different.

The polynucleotide vectors may be introduced into a suitable host cells using any suitable technique. For example, uptake of nucleic acids by mammalian cells is enhanced by several known transfection techniques for example electroporation or those including the use of transfection agents. Example of
10 these agents include cationic agents (for example calcium phosphate and DEAE-dextran) and lipofectants (for example lipofectam™ and transfectam™). Typically, the vectors are mixed with the transfection agent to produce a composition. It may be desirable to use an excess of the second vector in relation to the first vector. For example, the first and second vector may be mixed at a ratio of from
15 1:5 to 1:100 (first:second), more particularly from 1:10 to 1:50.

Preferred host cells are mammalian cells, such as rodent or human cells, and include CHO, BHK, COS and HeLa cells. Particular preferred cells are CHO cells. In a preferred embodiment, the host cells are initially deficient for the gene present in the second nucleotide sequence (e.g. dhfr). CHO cells deficient in dhfr
20 are described by Urlaub and Chasin, 1980, PNAS 77: 4216-4220 and are available from the ATCC as deposit ATCC CRL-9096. They have been deposited under the Budapest Treaty under deposit designation no. ATCC PTA-3672 at the American Type Culture Collection (ATCC), Rockville, Md. 20852, USA, on August 29, 2001. Examples of immortalized human cell lines which may be used with
25 the DNA constructs and methods of the present invention further include, but are not limited to, HT1080 cells (ATCC CCL 121), HeLa cells and derivatives of HeLa cells (ATCC CCL 2, 2.1 and 2.2), MCF-7 breast cancer cells (ATCC BTH 22), K-562 leukemia cells (ATCC CCL 243), KB carcinoma cells (ATCC CCL 17),
2780AD ovarian carcinoma cells (Van der Blick, A.M. et al., Cancer Res,
30 48:5927-5932 (1988), Raji cells (ATCC CCL 86), WiDr colon adenocarcinoma cells (ATCC CCL 218), SW620 colon adenocarcinoma cells (ATCC CCL 227), Jurkat cells (ATCC TIB 152), Namalwa cells (ATCC CRL 1432), HL-60 cells

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(ATCC CCL 240), Daudi cells (ATCC CCL 213), RPMI 8226 cells (ATCC CCL 155), U-937 cells (ATCC CRL 1593), Bowes Melanoma cells (ATCC CRL 9607), WI-38VA13 subline 2R4 cells (ATCC CLL 75.1), and MOLT-4 cells (ATCC CRL 1582), as well as heterohybridoma cells produced by fusion of human cells and 5 cells of another species. Secondary human fibroblast strains, such as WI-38 (ATCC CCL 75) and MRC-5 (ATCC CCL 171) may be used.

Transfections may conveniently be performed in microtitre plates, but other culture vessels may be used. Following transfection, cells are typically cultured in selection medium (i.e. medium that selects for cells that express the selectable 10 marker) to confluence and then in amplification medium containing a selection agent that causes amplification of the second nucleotide sequence, such as MTX. Typically, in the case of MTX, the agent is present in a concentration of about 48 nM (4.8×10^{-8} M).

Clones are then selected, typically grown in the same or similar 15 concentration of selection agent and screened for production of recombinant protein by, for example, ELISA and/or immunofluorescence. The highest producers are then selected, based on the results of the screen.

Ideally, the selected clones are then further tested to determine their growing properties, chromosomal stability, recombinant protein productivity and 20 recombinant protein activity.

Chromosomal stability can be determined for example by looking at cell morphology and/or performing an analysis of DNA content, such Coulter Counter nucleus DNA analysis. Generally, clones which have larger nuclei have a greater than diploid chromosome complement. These clones are likely to be less stable 25 and are generally rejected. Preferred clones are those which have a substantially identical nuclear DNA content to the parent host cell prior to transfection and selection.

Recombinant protein productivity can be tested by SDS-PAGE/Western blot of cells and/or culture supernatant where the protein is secreted (as is the case 30 for Epo). Testing may be conducted at a range of MTX (or other appropriate selection agent). It may be desirable to give consideration to not only the amount

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of protein but the pattern of protein (especially where multiple isoforms, such as differentially glycosylated isoforms, are produced, as is the case for Epo).

The method of the invention preferably further comprises one or more sub-cloning steps where selected clones are subjected to increasing concentrations of
5 the selection agent which causes amplification of the second nucleotide sequence. In the case of dhfr and MTX, typically initial cloning steps use 10^{-8} M concentrations of MTX, increasing up to 10^{-6} M concentrations.

Thus, in a further sub-cloning step, cell clones are test cultured in the presence of a range of concentrations of the selection agent (e.g. MTX) and the
10 recombinant protein titre determined. Sub-cloning is then preferably conducted at or above a concentration of the agent where the cell clones do not show a significant increase in protein production compared with lower concentrations. In the case of Epo and CHO cells, this is about 380 nM (3.8×10^{-7} M). In a preferred embodiment, the cells are cultured at progressively higher concentrations of agent
15 up to the highest desired concentration.

Sub-clones may then be further tested to determine their properties as described above. Optionally, further sub-cloning steps, typically with increased concentrations of selection agent (such as MTX), may be performed. In the Examples, a sub-cloning step is performed in the presence of $1.54 \mu\text{M}$ (1.54×10^{-6} M) MTX. In other cases the MTX concentration may be increased as high as or even higher than $10 \mu\text{M}$ (1×10^{-5} M).

Preferably, selected transformed host cells of the invention which express Epo or another desired polypeptide of interest, express Epo or said other polypeptide of interest at a rate of greater than $10 \mu\text{g}/10^6$ cells/day at an MTX
25 concentration of 7.7×10^{-7} M or 1.54×10^{-6} M, more preferably greater than 20, 25 or $30 \mu\text{g}/10^6$ cells/day. It is also preferred that the transformed host cells of the invention have a substantially identical nuclear DNA content to the parent host cell prior to transfection (e.g. a CHO dhfr- cell).

It may also be desirable to adapt host cells of the present invention to
30 serum-free media, typically after the final sub-cloning step. This may be achieved by cultivating seeded cells to confluence and replacing the growth medium with serum-free medium or gradually reducing serum concentration to zero over several

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passages. Preferably the cells are also adapted to growth as a suspension culture, optionally adherent to microcarrier beads.

Selected host cell-lines, i.e. the hybridoma or transformed host cells, or any mammalian cells from which the hybridoma or transformed host cells are derived, 5 may be cryopreserved prior to or after any cell culturing step, e.g. in liquid nitrogen using standard techniques. It is particularly preferred, however, that the freezing medium be as simple as possible, and most preferably consists of no more but water, a buffer, and at least one cryoprotective agent such as DMSO or PVP. In a highly preferred embodiment, the freezing medium, which may also be 10 applied as a medium for maintaining any of the aforementioned cells, comprises DMSO in combination with PVP.

Culture medium

The culture medium used in the method of the present invention for culturing mammalian cells does not contain serum. In particular, it is completely 15 free of components derived from an animal, such as proteins (including growth stimulating substances), amino acids, lipids and carbohydrates. Thus the components of the medium are mostly inorganic or synthetic and as such are not obtained directly from any animal source. Further, the culture medium is free of any functional recombinant proteins or polypeptide such as transferrin and insulin 20 or functional parts thereof.

The culture medium comprises water, an osmolality regulator, a buffer, an energy source, amino acids, a lipid source or precursor, a source of iron, non-ferrous metal ions and optionally one or more vitamins and cofactors.

Osmolality regulators are generally inorganic salts. Those which may be 25 used in the medium include NaCl, KCl, MgCl₂. It is advantageous to maintain osmolality in the range 200-450mOsm/Kg, preferably in the range 290-350mOsm/Kg.

Buffers are used in the medium to maintain the pH in the range 6.5 - 7.5 most preferably around pH 7.0. Suitable buffers include carbonates such as 30 NaHCO₃; also chlorides, sulfates and phosphates such as CaCl₂·2H₂O, MgSO₄·7H₂O, NaH₂PO₄·2H₂O, or sodium pyruvate; such buffers are generally

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present in an amount 0.05 to 5g/L, preferably 0.5 to 5g/L. For example about 2.5 g/L NaHCO₃ may be included.

Other buffers, such as N-[2-hydroxyethyl]piperazine-N min-[2-ethanesulphonic acid] otherwise known as HEPES and 3-[N-Morpholino]-propanesulfonic acid otherwise known as MOPS may also be used.

The term " amino acids" means that all 20 amino acids may be present. The amino acids are preferably of synthetic origin. The amounts which are usually included vary for each amino acid but are generally in the range 10 - 200 mg/l. However, L-glutamine is generally present at much higher concentration preferably in the range 1000-350 mg/l. Conveniently, the amino acid source may be based on a basal medium such as Dulbecco's modified eagle medium (DMEM) and/or Ham's F12. By adding amino acids to the basal medium - to supply for rapid consumption - an enriched amino acid supplemented medium is obtained.

The term "concentrate" describes a nutrient solution containing higher amounts of amino acids, and carbohydrates than the medium. Optionally one or more vitamin(s), lipid(s) and or trace element(s) can be added. The volume of the concentrate is typically 0.5 – 30 % of the starting volume. Most preferably between 1.25 – 5 %. It is added to supply for consumed nutrients.

The energy source of use in the medium is generally present in an amount of 0.5 to 10g/L and is preferably a monosaccharide such as mannose, fructose, galactose or maltose, most preferably glucose, particularly D-glucose. It is preferred that the initial concentration of glucose in the medium is at least 4 g/L.

The lipid source or precursor may, for example, be selected from lipid factors such as choline chloride, lipoic acid, oleic acid, phosphatidylcholine or linoleate and/or compounds involved in lipid production (lipid precursors), for example alcoholamines such as ethanolamine. It is preferred to include ethanolamine.

The iron source may be an inorganic or organic form and is typically present in an amount 0.025 to 0.5mg/L. Examples include ferric and ferrous salts such as ferric citrate or ferrous sulfate. The chelated salts such as ferric citrate and ferric ammonium citrate are preferred.

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Non-ferrous metal ions optionally of use in the medium include magnesium, copper and zinc; also sodium, potassium and selenium. It is preferred to include in the medium selenite ions, such as in the form of sodium selenite in an amount of 0.1 to 100 μ g/L, most preferably between 0.5 and 25 μ g/L.

5 Vitamins and enzyme co-factor vitamins (co-factors) optionally of use in the medium include Vitamin B6 (pyridoxine), Vitamin B12 (cyanocobalamin) and Vitamin K (biotin) present in an amount 0.001 - 1 mg/litre; Vitamin C (ascorbic acid) present in an amount 1 - 10 mg/litre, Vitamin B2 (riboflavin) present in an amount 0.1 - 1.0 mg/litre and Vitamin B1 (thiamine), niacinamide, Vitamin B5 (D 10 calcium pentothenate), folic acid, i-inositol generally present in an amount 2 - 20 mg/litre.

In large scale fermenters, mammalian cells are particularly susceptible to shear forces arising from the sparging of the vessel with gases and the mixing with the impeller. To minimize the occurrence of cellular damage it is preferred for 15 the medium to contain a cell protectant such as polyethylene glycol, polyvinyl alcohols or pluronic polyols. Preferably Lutrol® or Pluronic®-F68 (manufacturer: BASF) is used, typically at a concentration of about 0.05-0.3, most preferably of about 0.1%.

Host cells

20 Hybridoma or transformed host cells cultured according to the method of the invention are obtained from eukaryotic cells, generally mammalian cells such as rodent or primate cells. Preferred mammalian cells include mouse hybridomas, human/mouse heterohybridomas, SP2-0 cells, NS0 cells, baby hamster kidney cells, CHO cells, CHO dhfr⁻ cells, CHO-K1 cells, and recombinant cells obtained 25 from any one of these cell lines. Particularly preferred cells are CHO cells. In one embodiment, the host cells have been produced by transfecting a cell line initially deficient for a gene such as dhfr, which is amplified in response to selection pressure (see below) with a nucleotide sequence encoding said gene. CHO cells deficient in dhfr are described by Urlaub and Chasin, 1980, PNAS 77: 4216-4220 30 and are available from the ATCC as deposit ATCC CRL-9096. They have been deposited under the Budapest Treaty under deposit designation no. ATCC PTA-

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3672 at the American Type Culture Collection (ATCC), Rockville, Md. 20852,
USA, on August 29, 2001.

Transformed host cells and hybridomas of the present invention comprise a nucleotide sequence encoding a polypeptide of interest (POI). The POI is 5 preferably a glycosylated polypeptide, especially a glycosylated polypeptide which requires glycosylation which is only performed by mammalian cells. More preferably, the polypeptide is sialylated. A particularly preferred POI is selected from the group consisting of human growth hormone, human monoclonal antibodies, preferably of subclasses IgG, IgM, and IgA, and monoclonal 10 human/mouse chimeric antibodies. It is also preferred that the POI is secreted by the cell into the culture medium.

The coding sequence of the recombinant POI is operably linked to regulatory control sequences that direct expression of the POI in the host cell. Preferably, the nucleotide sequence encoding the POI is integrated into the 15 genome of the host cell. Various methods for introducing nucleotide sequences encoding POIs are known in the art - see for example *Sambrook et al., supra*.

In one embodiment, the nucleotide sequence encoding the POI is integrated into the genome of the host cell and operably linked to a second nucleotide sequence which when integrated into the host cell genome is amplified when the 20 host cell is contacted with a selection agent that causes amplification of the nucleotide sequence. Eukaryotic cells, in particular mammalian cells are capable of amplifying certain genes in response to a selective pressure, typically an enzyme inhibitor, resulting in an increase in the numbers of copies of that gene, which in turn increases levels of expression of the gene. The additional gene 25 copies may be maintained intrachromosomally, or in the form of extrachromosomal genetic material such as minute chromosomes (for review see Genes VI, Lewin, 1997, Oxford University Press, Oxford U.K., pp975-978). A well-characterized example is the dihydrofolate reductase (dhfr) gene which is amplified in response to methotrexate (MTX). Other examples include CAD gene 30 (encodes a protein which is involved in the first three steps of UMP synthesis) which is amplified in response to inhibitors of trans-carbamylase, and glutamine

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synthetase (see WO87/04462) which is amplified in response to methionine sulphoximine. Preferably, the second nucleotide sequence encodes dhfr.

Culture methods

According to the methods of the invention, the hybridoma or transformed host cells are cultured in the culture medium of the invention under conditions that allow expression of the POI. Eukaryotic cells, such as mammalian cells, may be cultured in a variety of number of formats and culture vessels. For example, cells may be cultured adherent to the bottom of plastic flasks or dishes, in suspension in stirred flasks/bioreactors, optionally adherent to solid carriers, preferably microcarriers such as polystyrol biobeads, or in roller bottle cultures. Since the objection of the present invention is to produce the POI in commercial quantities, it is preferred to grow the cells in bioreactors, particularly bioreactors which are capable of being batch fed and which have capacities of 4L or more.

Cells are typically seeded into the culture medium at a density of about 15 5×10^5 cells/mL. The optimum may vary for different cells types and can be determined by the skilled person. The pH is typically set at about pH 7.0, temperature to 37°C (although some cell lines such as insect cell lines are grown at lower temperatures) and pO₂ at about 50% air saturation.

Where the hybridoma or transformed host cells comprise integrated into their genome a dhfr (or equivalent) gene operably linked to the nucleotide sequence encoding the POI, a suitable amount of methotrexate (or equivalent) may be added to the medium. However, in a preferred embodiment, methotrexate is not added to the medium during production phase since we have found that in the absence of methotrexate, improved glycosylation patterns are obtained.

Cells are cultured typically for between about 7 to 14 days, depending on the cell line and recombinant POI. During that time, it is necessary to add fresh medium to prevent depletion of nutrients. Preferably, the culture time is extended by the addition of an enriched nutrient concentrate containing at least one amino acids, and a carbohydrate. In the concentrate, the amino acid is present in an amount of 0.05g/l to 50g/L, and the carbohydrate in an amount of 2 to 1000g/L.

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Most preferably, the amino acid is present in an amount of 0.5 to 5, and the carbohydrate between 25 to 75 g/L.

In a preferred embodiment of the invention, the concentration of the energy source, such as glucose is maintained at a concentration of at least 3g/L, typically 5 between 3g/L and 4g/L throughout the culture process.

In a further preferred embodiment, instead of keeping the pH constant at pH 7.0, the pH is initially greater than pH 7.0, such as pH 7.1, and is reduced to below pH 7.0, such as pH 6.9, during the culture process. Thus pH shifting from above to below neutral pH is used, provided that the pH is maintained within 10 limits tolerable to the host cells. Typically, the pH shift is performed over a period of time, such as at least 6 or 96 hours. Most preferably 72 hours.

Advantageously, the amount of recombinant POI produced by the cells is at least 200 mg/L, and preferably more than 300 or 400 mg/L.

The recombinant protein may be recovered from the culture supernatant or 15 a pellet of the cultured cells as appropriate. The recombinant protein is then typically subjected to one or more purification steps (see for example Broudy *et al.*, 1988, Archives of Biochemistry and Biophysics 265: 329-336; Ghanem *et al.*, 1994, Preparative Biochemistry 24(2): 127-142) such as affinity chromatography and/or ion-exchange chromatography.

20 Preferably in a composition comprising a polypeptide of interest obtained in a method according to the present invention the polypeptide is provided in a substantially pure state, such at least 90%, 95% or 99% pure.

In a preferred embodiment, the present invention may be performed using polynucleotide vectors and host cells in which there is

(a) a first polynucleotide vector comprising a first nucleotide sequence that encodes a polypeptide of interest, and, optionally, a second nucleotide sequence that encodes a selectable marker; and a second polynucleotide vector comprising a third nucleotide sequence that encodes a different selectable marker and that is free from a nucleotide sequence encoding a polypeptide of interest; or, alternatively,

(b) a polynucleotide vector comprising a first nucleotide sequence that encodes a polypeptide of interest, and, optionally, a second nucleotide sequence that

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encodes a selectable marker, and further a third nucleotide sequence that encodes a different selectable marker; and, optionally,

(c) at least one more polynucleotide vector comprising a nucleotide sequence encoding another polypeptide of interest and, optionally, a nucleotide sequence encoding a selectable marker;

the polynucleotide vectors being integrated into the genome of the hybridoma or transformed host cell.

Preferably the hybridoma or transformed host cell is derived from a mammalian cell, more preferably from a cell selected from the group consisting of rodent cells, particularly mouse hybridomas, human/mouse heterohybridomas, SP2-0 cells, NSO cells, baby hamster kidney cells, CHO cells, CHO dhfr⁻ cells, CHO-K1 cells, and recombinant cells obtained from any one of these cell lines.

Preferably the second nucleotide sequence encodes a dihydrofolate reductase polypeptide and the selection agent is methotrexate. Preferably the 5 polypeptide of interest is selected from the group consisting of human growth hormone, human monoclonal antibodies, preferably of subclasses IgG, IgM, and IgA, and monoclonal human/mouse chimeric antibodies.

The present invention is described further with reference to the following examples, which are illustrative only and non-limiting.

10

EXAMPLES

The principles of the present invention are hereinafter further described by way of examples, primarily relating to the expression of recombinant erythropoietin. They 15 are, however, similarly applicable to the expression of other recombinant proteins, particularly of those referred to herein and that are preferably selected from the group consisting of human growth hormone, human monoclonal antibodies, preferably of subclasses IgG, IgM, and IgA, and monoclonal human/mouse chimeric antibodies.

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Materials

Host cell line

Chinese hamster ovary (CHO) dihydrofolate-reductase deficient (ATCC CRL-9096). They have been deposited under the Budapest Treaty under deposit 5 designation no. ATCC PTA-3672 at the American Type Culture Collection (ATCC), Rockville, Md. 20852, USA, on August 29, 2001.

Cell culture Media

Protein-free supplement (100 x stock):

Ethanolamine (e.g. Sigma-Aldrich, Austria), 2.5×10^{-3} M;
10 Ferric-Citrate (e.g. Sigma-Aldrich, Austria), 2.5×10^{-2} M;
L-Ascorbic Acid (e.g. Sigma-Aldrich, Austria), 2.0×10^{-3} M;
Sodium Selenite (e.g. Sigma-Aldrich, Austria), 5.0×10^{-6} M;

Serum-free adaptation medium: 1:1 DMEM/Ham's F12, supplemented with:

L-glutamine, 6mM;

15 Serum-free production medium: 1:1 DMEM/Ham's F12, supplemented with:
protein-free supplement 1x; Lutrol® 0.1%; MTX 1.54×10^{-6} M,
Glucose 1g/l; NaHCO₃ 2.5g/l

Serum-free freezing medium: PBS, PVP-10, 20%; DMSO, 5%.

20 ***Methods in cell culture***

Growth of CHO dhfr

Dihydrofolate reductase deficient CHO cells (Urlaub et al., 1980, PNAS 77(7):4216-4220) (referred to as CHO dhfr) are cultivated in DMEM cultivation medium with a splitting ratio 1:10 twice a week.

25

Transfection of CHO cells

1-5x10⁴ cells per cm² are seeded in 25cm² T-flask bottles or 96-well plates the day before the lipofectin transfection is performed. The corresponding plasmids are mixed in the appropriate ratio, added to the lipofectin reagent

30 (GIBCO/BRL) according to the manufacturer's protocol (0.5-1 µl/cm²). Then the cells are overlaid with the transfection cocktail for four to sixteen hours in serum-free DMEM, before the DNA-containing medium is replaced with cultivation

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medium. After cultivation for 24 to 48 hours in the serum-containing medium the cells are switched to selection medium. Transfected cell pools are first cultivated in selection medium to confluence and then in amplification medium (4.8×10^{-8} M MTX) before screening the cell culture supernatants by ELISA for recombinant 5 protein production. Highest producers are determined, the MTX concentration increased two-fold and best producers used for further cultivation.

Analytical methods

ELISA detecting recombinant protein in different matrices

10 Antibodies

polyclonal serum: e. g., rabbit anti Epo biotinylated (R&D Systems; Catalog # AB-286-NA)

monoclonal antibody: e.g. mouse anti Epo (Genezyme; Cat. code AE7A5)

ELISA buffers

15 Coating buffer: 8.4 g/l NaHCO₃, 4.2 g/l Na₂CO₃, pH 9.6-9.8

Washing buffer: 0.2 g/l KCl, 1.15 g/l Na₂HPO₄ × 2H₂O, 0.2 g/l KH₂PO₄, 8 g/l NaCl, 0.1 % Tween 20

Dilution buffer: 2% PVP (Sigma Cat.No. PVP-4OT) in washing buffer

Sample buffer: 0.5% alpha mono-thio-glycerol in dilution buffer

20 Staining buffer: 7.3 g/l Citric acid × 2H₂O, 11.86 g/l Na₂HPO₄ × 2H₂O, pH 4.8-5.0
Staining solution: 100µl OPD solution/10 ml staining buffer

5µl H₂O₂/10 ml staining buffer

OPD stock-solution: PP: L0017

Method

25 The ELISA method used detects the expressed recombinant protein, e.g. Epo, in ng/ml concentration ranges, starting with 500 ng/ml and eight two-fold dilutions. One monoclonal antibody against the first 26 amino acids of Epo functions as a coating layer, binding Epo. In the next step Epo is specifically bound by the catcher antibody. Detection is arranged through a biotinylated Epo recognizing 30 rabbit antiserum. Visualization is performed by staining with OPD after streptavidin-peroxidase coupling to the plate.

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Immunofluorescence

Epo expressing CHO-cells are inoculated with 5×10^4 cells/200 μ l on a cover slip in a 6-well plate and incubated for 24-72 hours. The adherent cells are washed twice with PBS and fixed for 5 minutes with -20°C methanol, then air 5 dried and afterwards again soaked in PBS. Unspecific proteins are saturated by incubation with 20% FCS in PBS for 15 minutes and then anti-Epo is incubated for one hour. The reaction is visualized with anti-mouse IgG FITC conjugated. The fluorescence is detected by confocal microscopy at an excitation wavelength of 488 nm and an emission wavelength of higher than 515 nm.

10 Nucleus size determination

Materials

Coulter Counter® Model ZM (Coulter Electronics Inc.)

Coulter Channelyzer® Model 256

Incubation solution: Citric acid, 0.1M, Triton X 100, 2%

15 Electrolyte Isoton II (Kat-No.844 8011; Coulter Euro Diagnostic GmbH)

The Coulter Counter quantifies particles in size and number, that are suspended in an electric conductive fluid. The fluid is absorbed by vacuum through a capillary which carries electrodes on both sides. The change of the resistance induces a voltage impulse that can be digitized by the Coulter

20 Channelizer. Nucleus size correlates with DNA content of the cells, so that it is possible to distinguish between a diploid and a polyploid set of chromosomes.

Method

Approximately 1×10^6 CHO-cells are washed once in PBS, resuspended in 2 ml incubation solution and left there for 4-5 hours. The following steps are specific

25 for the Coulter Counter.

SDS Polyacrylamide-Electrophoresis and Western blotting

Materials

SDS Gels: Novex Tris-Glycine 4-20%

Sample buffer: Tris Glycine SDS 2x (Novex LC 2676)

30 Running buffer: Tris Glycine SDS (Novex LC 2675)

Blotting buffer: $\text{Na}_2\text{B}_4\text{O}_7 \times 10\text{H}_2\text{O}$, 50 mM, SDS, 0.1 %, methanol, 20%

Blotting matrix: PVDF Immobilon P 0.45 μ M Millipore; K8JM8238H

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- Washing buffer: see ELISA washing buffer
Dilution buffer: 1% milk powder in washing buffer
Detection buffer: NaCl, 0.1 M
Tris-HCl 0.1 M pH 9.5

5 Method

Epo containing samples are adjusted to 30 ng/20 μ l in 1x sample buffer with 1% α -MTG and applied to the SDS gel. At the end of the run, the proteins are blotted to a PVDF immobilon membrane for two hours and then the Epo is specifically stained with a monoclonal antibody detecting the first 26 amino acids of Epo.

- 10 Visualization is done with anti mouse IgG conjugated to alkaline phosphatase and NBT/BCIP staining.

Isoelectric focussing

Materials

System: Multiphor II, Pharmacia

- 15 IPG Gels: pH 2.5 – 7

Reswelling buffer: 9 g urea, 0.5 g CHAPS, 0.04 g DTE, 0.5 ml resolytes (pH 3.5-10)

10 μ l bromphenol-blue (0.1%), adjust to 25 ml with H₂O

Sample buffer: IPG sample buffer pH 3-10, 25 μ l in 625 μ l H₂O

- 20 Blotting buffer: 2.93 g Glycine, 5.81 g Tris, 20 ml methanol, 0.375 g SDS
adjust to 1000 ml with H₂O

Blotting matrix: PVDF Immobilon P 0.45 μ M Millipore; K8JM8238H

Washing buffer: see ELISA washing buffer

Dilution buffer: 1% milk powder in washing buffer

- 25 Detection buffer: NaCl, 0.1 M, Tris-HCl 0.1 M pH 9.5

Method

Epo containing samples are adjusted to 500-1000 ng/50 μ l, desalted, diluted 1:1 in sample buffer and applied to the reswollen IPG gel. Running conditions are first one minute 300V, then linear increase to 3500V and at the end 1 hour at 3500V.

- 30 During the whole focussing process a limit of 10 mA and 10W is set. Afterwards the proteins are blotted to a PVDF Immobilon membrane by diffusion overnight or by electroblot and then Epo is stained specifically with a monoclonal antibody

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detecting the first 26 amino acids of Epo. Visualization is done with anti mouse IgG AP conjugated and NBT/BCIP staining.

Determination of the DNA content

The DNA content of recombinant cell-lines is compared with the CHO-dhfr host
5 cell-line by FACS analysis.

Materials

Washing-Buffer: 0.1 M Tris-HCl, pH 7.4, 2 mM MgCl₂, 0.1% Triton X100

Staining Buffer: 0.3 µg/ml DAPI (Hoechst) in washing buffer

Method

10 5x10⁵ cells are washed in PBS and fixed with ice cold 70% ethanol. Afterwards the cells are washed twice with washing buffer and then incubated in staining buffer for 30 minutes at RT. The DNA content is measured with the FACS Vantage (Becton and Dickinson) at 359 nm excitation and 450 nm emission.

In-vitro specificity test

15 The human erythroleukemic cell-line TF-1 (German Collection of Microorganisms and Cell Cultures) is growth-dependent on IL3 or hGM-CSF. These cytokines display synergistic effects on proliferation, while Epo can maintain the viability for some time. The cells are routinely grown in GM-CSF and Epo containing cultivation medium.

20 **Test Supplements**

Cultivation medium: RPMI 1640, supplemented with 4mM Gln, 10% FCS,
20µg/ml transferrin, 10µM beta- mercapto-ethanol,
12ng/ml rhGM-CSF, 3U/ml rh Epo,

25 Test medium: RPMI 1640, supplemented with 4mM Gln, 100µg/ml
transferrin, 2mg/ml BSA

Methods

The functionality test is performed as a MTT viability test in 96-well plates (Hammerling *et al.*, 1996, J Pharm Biomed Anal 14(11):1455-69). Samples are

30 diluted 1:2 fold eight times, starting with 100 ng Epo per ml in test medium. 50 µl of each sample dilution, standard dilution or blank are transferred to the 96-well testing plate. TF-1 cells are washed three times with cold PBS and adapted to

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2x10⁵ cells per ml in test medium. Each well of 96-well test plate is overlayed with 50 µl of the cell suspension and these cells are left for 72 hours in the CO₂ incubator. Afterwards 10 µl MTT solution (6 mg/ml in PBS) are added and incubated at 37 °C for 4 hours. The dye is dissolved with 100 µl SDS/HCl (10% SDS in 0.1 M HCl) for another 4 hours in the dark and the Epo dependent viability is photometrically determined at 550/690 nm.

Example 1 - Construction of plasmid Epo/neo

1. Construction of p2-neo

10 1.1 Preparation of the vector fragment from pSV2neo containing the SV40 early promoter

The basis of the vector construction is the pBR322 plasmid backbone contained in pSV2neo. The smaller *Eco*RI – *Pvu*II restriction fragment includes this pBR322 backbone and the neighboring *Pvu*II – *Hind*III fragment from SV40 bears the 15 relevant fragment of the SV40 early promoter.

Plasmid pSV2neo (ATCC 37149) is cut with the restriction enzymes *Eco*RI and *Hind*III. The two resulting fragments has a sizes of 3092 bp and 2637 bp. The 2637 bp fragment consists of an *Eco*RI – *Pvu*II restriction fragment including a pBR322 backbone and a neighboring *Pvu*II – *Hind*III fragment which contains a 20 fragment of the SV40 early promoter. The 2637 bp is prepared and purified via gel electrophoresis.

1.2 Preparation of the neomycin resistance gene

The neo gene is taken from the transposon Tn5 of pSV2neo. It is amplified as a fragment containing solely the coding region of the gene. As part of the cloning 25 strategy, recognition sites for restriction endonucleases are introduced at both ends. A *Hind*III site is built in the upstream amplification primer, an *Eco*RI and a *Spe*I site in the downstream primer. The amplified region corresponds to nucleotides 2846 to 1938 in the sequence of pSV2neo (Genbank Accession No. U02434). The oligonucleotides are designed as follows:

- 30 -

5 Oligo 2004-01: length: 38mer

5' - ggg gga agc ttg ttg gga agc cct gca aag taa act gg - 3'

5' HindIII: aagctt - g (= pos. 2846 in pSV2neo) ttgggaagccctg.....

5 Oligo 2004-02: length: 42mer

5' - ggg gaa ttc act agt gag tcc cgc tca gaa gaa ctc gtc aag - 3'

5' EcoRI/SpeI:

gaa ttc actagt - g (= pos. 1938 in pSV2neo)

agtcccgctcagaa.....

10

The amplification product of primers 2004-01 and 2004-02 is prepared by PCR using *Pwo* polymerase (Roche Diagnostics). The parameters for the process are:

20 ng of pSV2neo, 10 pmol of each primer, 10 mmol dNTPs, 2.5 U *Pwo*

polymerase in the supplied buffer to a total volume of 50 µl; temperature profile: 5

15 min 95 °C, 35 times (30 sec 95 °C, 20 sec 65 °C, 90 sec 72 °C), 3 min 72 °C, cooling at 4 °C until further use.

The resulting DNA fragment of 935 bp is purified by DNA isolation columns (Mini, Wizard Promega GmbH), digested with *Eco*RI and *Hind*III, and purified via an agarose gel and eluted using Spin Columns (Supelco).

20 1.3 Construction of p1-neo

The amplified *Eco*RI-*Hind*III neo gene fragment is ligated to the *Eco*RI-*Hind*III vector fragment from pSV2-neo using Ligation Express (Clontech) and transformed into an *E.coli* host (*E.coli* SURE (Stratagene)). Transformants are selected by growth on LB medium supplemented with 50 mg/l ampicillin.

25 Plasmid DNA is isolated from clones and checked by restriction analysis using *Eco*RI plus *Nco*I (3 fragments of 2527 bp, 780 bp and 251 bp, respectively). Plasmid DNAs showing the expected fragments are further checked by sequencing relevant parts of the constructs. A plasmid DNA containing a verified SV40early promoter and neomycin resistance gene is designated as p1-neo.

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1.4 Preparation of the SV40 termination region SV40LTpolyA/IVS

PCR primers are designed to amplify a fragment (nucleotides 751 to 1598) of the SV40 termination region present in pSV2neo. The upstream primer also contains a restriction site for *Spel*. In addition to the *BamHI* site already included
5 at position 751 of pSV2-neo an *EcoRI* site is introduced into the downstream primer separated by a 6 nucleotide spacing region from *BamHI*. The sequences of the two primers are as follows:

Oligo 2004_05: length: 40mer

10 5'- ggg gac tag ttt gtg aag gaa cct tac ttc tgt ggt gtg a - 3'
5' *Spel*: actag - t (= pos. 1598 in pSV2neo) ttgtgaaggag.....

Oligo 2004-06: length: 46mer

5' - ggg gga att cgg agg ggg atc cag aca tga taa gat aca ttg atg a - 3'
15 5' *EcoRI/BamHI*: gaattc - g (= pos. 751 in pSV2neo)gatcc
agacatgataag.....

The amplification product of primers 2004-05 + 2004-06 is prepared by PCR using *Pwo* polymerase (Roche Diagnostics) as described above. The resulting
20 DNA fragment of 873 bp is purified using DNA isolation columns, digested with *EcoRI* and *Spel* and gel-purified.

1.5 Preparation of p2-neo

p1-neo plasmid DNA is digested using *EcoRI* + *Spel*. The resulting linearized fragment is purified, ligated with the amplified fragment containing
25 SV40LTpolyA/IVS and transformed into an *E.coli* host. Transformants are selected by growth on LB medium supplemented with 50 mg/l ampicillin.

Plasmid DNA is isolated from clones and checked by restriction analysis using *EcoRI* (1 fragment of 4411 bp) and *Ncol* (2 fragments of size 3631 bp and 780 bp) and *SphI* (3 fragments of size 3499 bp, 840 bp and 72 bp). Plasmid
30 DNAs showing the expected fragments are further checked by sequencing relevant parts of the constructs. A plasmid DNA containing a verified SV40LTpolyA/IVS is designated as p2-neo.

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2. Construction of plasmid p3

2.1 Preparation of the SV40 early promoter fragment

Plasmid pSV2neo is used as a source for the SV40 early promoter fragment. The fragment size is almost identical to the one used in constructing the p2 plasmids.

5 However the ends of the fragment are modified to introduce recognition sites for *Bam*HI and *Not*I. The oligonucleotide primers used to amplify the promoter are designed as follows:

Oligo 2004-07: Length: 38mer

10 5' - ggg ggg atc ctg tgg aat gtg tgt cag tta ggg tgt gg - 3'
5' *Bam*HI: ggatcc - t (= pos. 3435 in pSV2neo) gtggaat.....

Oligo 2004-08: Length: 46mer

5' - ggg ggc ggc cgc agc ttt ttg caa aag cct agg cct cca aaa aag c - 3'
15 5' *Not*I: gcggccgc - a (= pos. 3093 in pSV2neo) gcttttgcaaaag.....

The amplification product of primers 2004-07 + 2004-08 is prepared by PCR using *Pwo* polymerase (Roche Diagnostics), as described above. The resulting DNA fragment of 365 bp is purified using DNA isolation columns, digested with 20 *Bam*HI and *Not*I, and gel-purified.

2.2 Preparation of the pBluescript vector part

pBluescript II SK + DNA is sequentially restricted using *Bam*HI and *Not*I, respectively. The DNA is dephosphorylated using alkaline phosphatase. The *Bam*HI/*Not*I fragment is purified from the small fragment via agarose gel 25 electrophoresis prior to ligation.

2.3 Preparation and verification of plasmid p3

The amplified *Bam*HI-*Not*I fragment containing the SV40 early promoter is ligated into the prepared pBluescript II SK + vector using T4 DNA Ligase (Promega GmbH). Plasmid DNA from *E.coli* SURE (Stratagene) transformants is isolated and 30 purified from colonies on LB medium supplemented with 100 mg/l ampicillin.

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Resulting DNAs are checked by restriction analysis using *Eco*RI plus *Nco*I (2 fragments of size 3039 bp and 253 bp).

Two plasmid DNAs showing the expected fragments are further checked by sequencing. Both strands of the SV40 early promoter are sequenced so that each 5 position could be verified. The plasmid is designated as p3.

3. Isolation of human Epo cDNA

3.1 Isolation of total RNA with TRIzol® Reagent

TRIzol® reagent, used for isolation of Epo RNA from human kidney tissues 10 (obtained from the Lainzer Krankenhaus hospital) is a mono-phasic solution of phenol and guanidine isothiocyanate. During cell lysis guanidine isothiocyanate forms a water-soluble complex with RNA while cells are disrupted. Addition of chloroform, followed by centrifugation, separates the solution into an aqueous, RNA containing and an organic phase. After separation of the aqueous phase the 15 RNA is precipitated with isopropyl alcohol, washed with ethanol, air-dried and resuspended in RNase free water.

Human kidney tissue fragments are cut into small pieces, forced through a 100 µm cell strainer, centrifuged (179xg/10 min) and the resulting pellet is washed three times with PBS. Then the pellet is resuspended in PBS, aliquoted in 20 sterile tubes, frozen to -196°C and stored at -80°C until further use.

The frozen tissue is lysed by addition of 1 ml TRIzol® reagent, homogenized and incubated at 15-30°C for 5 minutes to ensure complete dissociation. After addition of 200 µl chloroform, shaking the tube and incubation for 2-3 minutes at 15-30°C the tube is centrifuged at 12000xg for 10 minutes. Following 25 centrifugation, the upper aqueous phase is carefully transferred to a fresh tube mixed with 500 µl isopropyl alcohol and incubated at 15-30°C for 10 min. The precipitated RNA is centrifuged (12000xg, 10 min), the pellet washed with ethanol, centrifuged again, air dried and dissolved in RNase free DEPC water. Total RNA content is measured photometrically at 260 nm.

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By evaluating the ratio of OD_{260nm} and OD_{280nm} (maximum absorbance of proteins) one can estimate the purity of the RNA isolation. It should range between 1.6 and 1.8.

3.2 mRNA isolation with Dynabeads Oligo (dT)25

5 Dynabeads Oligo (dT)₂₅ mRNA DIRECT kit employs hybridization of the polyadenosine tail RNA of eukaryotic mRNA to supermagnetic, polystyrene particles containing 25 nucleotide long chains of deoxy-thymidylate covalently attached to their surface. mRNA bound to the magnetic beads can be separated using a Dynal magnetic particle concentrator (Dynal MPC®).

10

<i>Washing buffer</i>	Tris-HCl 10 mM, pH 8,0; LiCl 0,15 mM; EDTA 1mM
<i>2 x Binding buffer</i>	Tris-HCl 20 mM, pH 7,5; LiCl 1 mM; EDTA 2 mM

For 10 µg of total RNA, 100 µl Dynabeads oligo (dT)₂₅ are separated in the Dynal MPC® and washed twice with 2x washing buffer. Meanwhile total RNA is adjusted to a volume of 200 µl with 1x washing buffer and denatured by incubation at 65 °C for 4 minutes. Then the RNA is mixed with the beads, incubated at room temperature for 5 minutes and separated in the Dynal MPC®. The beads are washed twice with 1x washing buffer. The polyadenylated RNA is 20 eluted from the Dynalbeads Oligo (dT)₂₅ by incubation with elution buffer (2 x 10 µl) for 4 minutes at 65 °C. Dynabeads are separated in the Dynal MPC® and the supernatant is immediately transferred to a new RNase free microcentrifuge tube. The eluate is used directly for reverse transcription.

3.3 Reverse Transcription

25 The specific primer for Epo is denatured by 4 min incubation at 80 °C and the mRNA is denatured by 5 min incubation at 65 °C. The following components are added to a sterile 1.5 ml microcentrifuge tube on ice:

30

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Reagent	Final concentration
mRNA	10 µl
MMLV (200 U/µl)	0,25 µl
Boehringer PCR buffer (10 x)	2 µl
dNTPs (10 mM)	2 µl
MgCl ₂ (50 mM)	1 µl
Epo for (100 pmol/µl)	1 µl
DTT (0,1 M)	0,25 µl
RNase inhibitor (40 U/µl)	0,25 µl
H ₂ O	3,25 µl

Incubation: 60 min 37 °C

Inactivation: 5 min 100 °C

3.4 Polymerase chain reaction

- 5 The following components are added to a sterile 1.5 ml microcentrifuge tube at 4 °C. PCR conditions are listed below.

Reagent	Epo
Template	cDNA Epo 5 µl
polymerase	Vent 1U (0,5µl)
polymerase buffer (10x)	Vent buffer 1x (10µl)
dNTPs (10 mM)	200 µM (2µl)
MgCl ₂ (50 mM)	/
Primer for (10 pM)	30pM (3µl)
Primer back (10 pM)	30 pM (3µl)
DMSO	76,5 µl
H ₂ O	

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PCR cycle		
1 Denaturation	95 °C	2 min
2 Denaturation	94 °C	45 sec
3 Primer annealing	58 °C	30 sec
4 extension	72 °C	1 min
5. finish extension	72 °C	10 min
6. cycles	30	

The PCR amplification products are analyzed by agarose gel electrophoresis.

3.5 Agarose gel electrophoresis

5

6 x BX buffer bromphenol blue 0,25 %; xylene cyanol 0,25 %, glycerol 30%

TAE buffer Tris base 242g; glacial acetic acid 57,1 ml; EDTA (0,5 M, pH 8,0) 100 ml; adjusted to 1000 ml H₂O

Lambda-Marker III 10 µg bacteriophage Lambda-wildtype-Dann

10 (2,5 µl Hind III + 2,5 µl Eco R I + 20 µl buffer R (Fermentas) filled up with H₂O ad 200 µl; 1 h at 37°C digested, 20 min at 65 C inactivated; supplemented with 40 µl BX-loading buffer)

15 1g agarose and 99g 1xTAE buffer are melted in the microwave oven, cooled down to approximately 60°C and supplemented with 3 µl of ethidium bromide stock solution (10 mg/ml). Gels are run in 1xTAE buffer at 100-300 V for approximately 30 min, depending on the length of the DNA fragments to be separated. Each lane contains 10 µl sample mixed with 2 µl 6 x BX buffer.

20 Identification of DNA fragments is based on comparison with a Lambda/Hind III digest molecular weight standard.

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3.6 Preparation of PCR products and vectors for ligation

3.6.1 Restriction of Vector DNA and insert for sticky end cloning

10u of restriction enzyme and appropriate restriction buffers are mixed with 1 μ g vector DNA and insert according to manufacturers instructions. The mixture 5 is incubated at 37°C (30°C for SmaI) between 30 and 60 min, depending on the enzymes used, vector and insert. Then the enzyme is inactivated by heating up to 65°C for 10 min and the reaction mixture is analyzed by agarose gel electrophoresis.

3.6.2 Ligation

10 *pIRESneoSV40 vector*

pIRESneo vector (Clontech laboratories) contains the internal ribosome entry site (IRES) of the encephalomyocarditis virus (ECMV), which permits the translation of two open reading frames from one messenger RNA. The expression cassette of *pIRESneo* contains the human cytomegalovirus (CMV) major 15 immediate early promoter/enhancer followed by a multiple cloning site (MCS), the ECMV IRES followed by the neomycin phosphotransferase gene and the polyadenylation signal of the bovine growth hormone. In this vector the CMV promoter is replaced by the SV40 early promoter.

20 Vector and PCR product are ligated with T4 DNA ligase. For optimal ligation approximately 20 ng vector and 200 ng insert (depending on the length) are used in a molar ratio of about 1:10 and mixed with following reagents in a total volume of 10 μ l H₂O. The incubation is performed overnight at 15°C and 3 h at RT. Then the ligase is heat-inactivated by incubation at 65°C for 10 minutes.

Reagent	Final amount
Vector (<i>pIRESneoSV40</i>)	~ 20 ng ~ 200 ng
Insert (Epo)	1U (1 μ l)
T4 DNA Ligase	1x (2 μ l)
Buffer (5x)	ad 10 μ l
H ₂ O	

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3.6.3 Bacteria and culture media

JM109

(Promega, USA)

LB-medium

peptone from casein 10 g; yeast extract 5 g; NaCl 10 g,
adjusted to 1000 ml with H₂O and set to pH 7,0 with 5M

5

NaOH

LB agar

15 g agar in 1000 ml LB-medium

LB-Amp

100 µl ampicillin (100 mg/ml) in 1000 ml LB-medium

SOC medium

bacto tryptone 20 g; yeast extract 5 g; NaCl 10 mM; KCl 3
mM; MgCl₂ 10 mM; glucose 20 mM, MgSO₄ 10 mM

10 3.6.4 Transformation using CaCl₂

Preparation of competent bacteria (JM109)

10 ml of LB medium is inoculated with *E.coli* (JM109) and grown overnight at 37°C. 4 ml bacterial culture is diluted 1:100 in LB medium and grown until having reached OD_{260 nm} of 0.8. Bacteria are centrifuged at 4500 rpm for 10 min at 15 4°C and the cell pellet is resuspended in 10 ml 0.1 M CaCl₂ (4°C)/50 ml bacterial suspension used. The cells are centrifuged, the pellet is resuspended in 2 ml 0.1 M CaCl₂ and aliquoted to a total volume of 100 µl, frozen in liquid nitrogen and stored at -80°C.

Transformation

20 In pre-chilled 17x100 mm polypropylene culture tubes 5-10 ng plasmid DNA is added to JM109 competent bacteria, gently mixed and put on ice for 30 min. Then the cells are heat-shocked for 45 seconds in a waterbath at exactly 42°C without shaking and immediately placed on ice for 2 minutes. Then 900 µl SOC medium is added to the tube and incubated for 30 min at 37°C before plating 100 25 µl of bacteria suspension on LB-Amp plates.

3.6.5 Screening and establishing glycerin cultures

Ampicillin resistant colonies are screened for the inserted DNA fragment by PCR technique. Portions of ampicillin resistant colonies are mixed with the PCR 30 reaction mixture and with specific primers against the cloned DNA fragment (see below). Positive colonies show PCR-amplified DNA bands in agarose gel

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electrophoresis. These colonies are then propagated in LB-Amp medium for further analysis and plasmid purification. For further use and storage 1 ml of desired bacteria culture is mixed with 500 μ l glycerin (87 %) and stored at -80°C.

3.6.6 Wizard® Plus SV Minipreps DNA Purification System

5 *Cell resuspension solution* Tris-HCl 50 mM, pH7.5; EDTA 10 mM, RNase A 100 μ g/ml

Cell lysis solution NaOH 0.2 M, SDS 1%

Neutralization solution guanidine hydrochloride 4.09M, potassium acetate 0.759M; glacial acetic acid 2.12M, pH 4.2

10 *Column wash solution* potassium acetate 60 mM, Tris-HCl 10 mM, pH 7.5; ethanol 60%

2-3 ml LB-Amp medium are inoculated with a single colony and incubated at 37°C over night. The solution is centrifuged (12000xg, 5 min) and the resulting pellet is thoroughly resuspended in 250 μ l resuspension solution and then 250 μ l 15 of cell lysis solution, mixed by inverting the tubes 4 times and incubated at RT for 1-5 min. Thereafter 10 μ l of alkaline protease solution (incubated at RT for 5 min) and 350 μ l neutralization solution were added. The tube is immediately mixed by inverting it 4 times and the bacterial lysate is centrifuged at 12000xg for 10 min at RT. The cleared lysate is transferred to Spin Columns and centrifuged 20 (12000xg, 5 min) and the column is washed twice with washing solution (750 μ l/250 μ l). The DNA is eluted with 100 μ l nuclease-free water.

3.6.7 Sequencing of plasmids

The inserted sequences are sequenced by IBL (Gerasdorf, Austria) and by GenXpress (Maria Wörth, Austria) with specific primers. Oligonucleotide primers 25 for the amplification of Epo and SV40early promoter and for sequence analysis are listed below.

- 40 -

Oligonucleotide primer	Sequence
<i>SV40early promoter</i>	
SV40 early Clal for	5`-aga tcg atc aag ctt ttt gca aaa gcc tag-3`
SV40 early Nrul back	5`-agt cgc gag cgc agc acc atg gcc tg-3`
SV40 early 281 back	5`-gcc cag ttc cgc cca ttc-3`
<i>Epo</i>	
Epo BamHI for	5`-tag gat cct cat ctg tcc cct gtc ctg c-3`
Epo EcoRI back	5`-tag aat tcc gcc atg ggg gtg cac gaa tgt cc-3`
Epo 221 for	5`-taa ctt tgg tgt ctg gga-3`
Epo 204 back	5`-tcc cag aca cca aag tt-3`
<i>pIRESneo</i>	
pIRESneo 181 back	5`-tta ggg tta ggc gtt ttg cg-3`
pIRESneo 1016 for	5`-act cac ccc aac agc cg-3`
pIRESneo 2786 for	5`-ggcc aaa caa cag atg gct-3`
pIRES-200 back	5`-tgg aaa gag tca aat ggc-3`

4. Construction of plasmid p5

4.1 Preparation of the Epo gene fragment

The structural gene for Epo (human erythropoietin) is amplified by PCR using pSVGPIRNEO as a template DNA. The sequence of Epo is given in GenBank Accession No. M 11319.1. Recognition sites for *NotI* and *KspI* are introduced into the upstream and downstream primer, respectively. The primers are designed as follows:

Oligo 2004-09: Length: 45mer

10 5`- ggg ggc ggc cgc atg ggg gtg cac gaa tgt cct gcc tgg ctg tgg - 3`
5' *NotI*: gcggccgc a (= pos. 665 in PSVGPIRNEO) tgggggtg.....

Oligo 2004-10: Length: 44mer

5`- ggg gcc gcg gtc atc tgt ccc ctg tcc tgc agg cct ccc ctg tg - 3`
15 5' *KspI*: ccgcgg - t (= pos. 1246 in PSVGPIRNEO) catctgtccccct.....

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The amplification product of primers 2004-09 + 2004-10 is prepared by PCR using *Pwo* polymerase (Roche Diagnostics), as described above. The resulting DNA fragment of 604 bp is purified using DNA isolation columns, digested with *Kspl* and *NotI*, and gel-purified. The resulting 592 bp *Kspl/NotI* Fragment is used
5 in the triple ligation described below.

4.2 Preparation of the termination region SV40LTpolyA/IVS

The termination region of SV40LTpolyA/IVS is recloned from pSV2neo by PCR in a similar manner to that described above in section 1.4 for the construction of p2-neo except that the primers are designed with different
10 restriction endonuclease recognition sites: the site for *Kspl* (= *SacII*) is included into the upstream primer and the sites for *SacI* and *EcoRI* into the downstream primer.

Oligo 2004-11: length: 42mer

15 5' - ggg gcc gcg gtt tgt gaa gga acc tta ctt ctg tgg tgt gac - 3'
5' *Kspl*: ccgcgg - t (= pos. 1598 in pSV2neo) ttgtgaaggaa.....

Oligo 2004-12: length: 46mer

5' - ggg gga gct cga att cga tcc aga cat gat aag ata cat tga g - 3'
20 5' *SacI/EcoRI*: gagctc gaattc - g (= pos. 752 in pSV2neo)
atccagacatg.....

The amplification product of primers 2004-11 + 2004-12 is prepared by PCR using *Pwo* polymerase (Roche Diagnostics), as described above. The resulting
25 DNA fragment of 873 bp is purified using DNA isolation columns and digested with *Kspl* and *SacI*. The resulting DNA fragment of 858 bp is then gel-purified.

4.3 Preparation of the p3 vector part

p3 plasmid DNA is sequentially digested using *NotI* and *SacI*, respectively. The DNA is treated with alkaline phosphatase and the vector fragment is gel-
30 purified.

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4.4 Triple ligation and isolation of plasmid p5

The *NotI/SacI* vector part of plasmid p3, the *KspI/NotI* Epo gene and the *KspI/SacI* termination region SV40LTpolyA/IVS are ligated in one ligation reaction (Ligation Express, Clontech). Transformants of are selected on LB medium

5 supplemented with 100 mg/l ampicillin.

Positive transformants containing both fragments inserted are screened by colony hybridization using both amplified fragments 2004-09/2004-10 and 2004-11/2004-12, as labeled probes. Ten clones which gave a positive hybridization signal with both probes are chosen for a "midi" scale plasmid preparation

10 (Qiagen).

Restriction analysis is performed using the enzymes *BamHI* (1 fragment 4723 bp), *EcoRI* (2 fragments, 2913, 1810 bp) and *PvuII* (4 fragments 2513, 1204, 903, 103 bp). Two clones showing the correct restriction fragments are selected and checked by sequencing. The whole cassette cloned into pBluescript

15 II SK + is sequenced and compared to the expected nucleotide sequence. Every single nucleotide could be successfully verified. The plasmids are designated p5.

5. Construction of pEpo/neo

5.1 Construction of pEpo/neo 12-1

20 p5 plasmid DNA is digested with *BamHI* and *EcoRI* and the resulting 1792 bp fragment representing the cassette of SV40promoter-Epogene-SV40terminator is gel-purified.

Plasmid p2-neo is also digested with *BamHI* and *EcoRI* and the linearized vector 25 gel-purified. Additionally the DNA is dephosphorylated using alkaline phosphatase and purified with Amicon Micropure enzyme removers.

Both fragments, the 4411 bp p2-neo vector and the 1792 bp cassette from p5, are ligated (Ligation Express, Clontech) and transformed into *E.coli* SURE. Plasmid DNA is isolated from various transformants grown on LB medium supplemented with 70 mg/l ampicillin and analyzed by digestion using restriction 30 endonucleases *PvuII*, *EcoRI* and *Ncol*.

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A clone showing the expected fragments (*Eco*RI: 6191 bp, *Nco*I: 4085, 1326 and 780 bp, *Pvu*II: 3273, 2130, 685 and 103 bp) is selected and designated as pEpo/ neo-12.

For additional purification the DNA is retransformed into *E.coli* SURE (see 5 above) and plasmid DNA prepared using a "Midi-prep" procedure (Qiagen) from a culture inoculated by a single colony (pEpo/neo-12-1). Restriction analysis is performed using the following enzymes: *Bam*HI, *Hind*III, *Eco*RI, *Nco*I, *Not*I, *Pst*I, *Spe*I, *Sph*I, *Pvu*II, *Nar*I. The expected fragments and sizes could be found, verifying the clone as a correct pEpo/neo clone.

10 5.2 The final construction of pEpo/neo

The upstream region of the Epo gene in pEpo/neo-12-1 is changed at position minus-3 from the start ATG. An additional nucleotide A is introduced to result in the purine base G at position -3 from start ATG. A purine at that position may improve the expression level of the gene. For that purpose the Epo gene is 15 reamplified using an adapted upstream primer 2004-09-a:

Oligo 2004-09-a: length: 46mer

5' - gggggcggccgcaatgggggtgcacgaatgtcctgcctggctgtgg - 3'

20 The amplification product of primers 2004-09_a + 2004-10 is prepared by PCR using *Pwo* polymerase (Roche Diagnostics), as described above. The resulting DNA fragment of 605 bp is purified using DNA isolation columns and digested using *Ksp*I and *Not*I. The resulting DNA fragment of 593 bp is then gel-purified.

pEpo/neo-12-1 plasmid DNA is digested with *Ksp*I and *Not*I, respectively, to 25 remove the Epo gene. The 5599 bp fragment is then gel-purified. Both prepared DNAs are ligated to each other (Ligation Express, Clontech). Plasmid DNA from transformants is isolated and purified from colonies on LB medium supplemented with 70 mg/l ampicillin. DNAs are analyzed by restriction using *Nco*I in a first screening.

30 A positive clone is selected to isolate DNA using a "Midi prep" procedure (Qiagen). An extended restriction analysis is performed using *Bam*HI, *Hind*III,

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EcoRI, Ncol, NotI, PstI, Spel, SphI, PvuII, NarI. The expected fragments and sizes could be found, verifying the clone as a correct pEpo/neo. Every single nucleotide of the whole cassette (SV40early_promoter – **neo gene** – SV40LTpolyA/IVS – SV40early_promoter – **Epo gene** – SV40LTpolyA/IVS) inserted in the pBR322 vector-part is also confirmed by sequencing.

Example 2 - Construction of plasmid Epo/dhfr

1. Construction of p2-dhfr-CDS

1.1 Preparation of the dhfr gene

The dhfr gene used for the vector construction is taken from a mouse cDNA, present in plasmid pLTRdhfr26 (ATCC 37295). The nucleotide sequence of the mouse dhfr cDNA (MUSDHFR) is available as GenBank Accession No. L26316.

The dhfr is amplified from pLTRdhfr26 using primers designed to produce a fragment containing the coding region from the start ATG at position 56 to the stop codon TAA at position 619. As for the amplification of the neomycin resistance gene described above, *HindIII* and *Spel* sites are introduced in the upstream and downstream amplification primers, respectively. An *EcoRI* site is also introduced into the reverse primer beside the *Spel* site. The sequence of the oligonucleotides is as follows:

20

Oligo 2004-13: length: 39mer

5' - ggg gaa gct tat ggt tcg acc att gaa ctg cat cgt cgc - 3'

5' *HindIII*: aagctt - A (= pos. 56 in MUSDHFR) TGgttcgaccattg.....

25 *Oligo 2004-14:* length: 42mer

5' - ggg gaa ttc act agt tag tct ttc ttc tcg tag act tca aac - 3'

5' *EcoRI* / *Spel*:

gaattc actag - t (= pos. 619 in MUSDHFR)

tagtctttcttcgttagactcaaact.....

30

The amplification product of primers 2004-13 + 2004-14 is prepared by PCR using *Pwo* polymerase (Roche Diagnostics), as described above. The resulting

- 45 -

DNA fragment of 588 bp is purified using DNA isolation columns, digested with *Hind*III and *Eco*RI and gel-purified.

1.2 Preparation of p1-dhfr-CDS

The amplified *Eco*RI-*Hind*III dhfr gene fragment is ligated to the *Eco*RI-
5 *Hind*III vector fragment from pSV2-neo using Ligation Express (Clontech), and transformed into an *E.coli* host. Transformants are selected by growth on LB medium supplemented with 50 mg/l ampicillin. Plasmid DNA from transformants is isolated and purified from colonies on LB medium supplemented with 50 mg/l ampicillin.

10 Plasmid DNA is isolated from clones and checked by restriction analysis using *Eco*RI plus *Scal* (3 fragments of size 2225 bp, 514 bp and 473 bp).

Plasmid DNAs showing the expected fragments are further checked by sequencing relevant parts of the constructs. A plasmid DNA containing a verified SV40early promoter and dihydrofolate reductase gene is designated as p1-dhfr-
15 CDS. The analysis of the sequences revealed one deviation within the dhfr gene from the sequence published in MUSDHFR, specifically a change from T to C at position 451 of the MUSDHFR sequence. Subsequent sequencing showed that this change is also present in the source plasmid. However the resulting change does not cause a change in the amino acid sequence encoded by nucleotide
20 sequence since CTT and CTC both encode leucine.

1.3 Preparation of p2-dhfr-CDS

p1-dhfr-CDS plasmid DNA is digested using *Eco*RI + *Spe*I. The resulting linearized fragment is purified and ligated with the amplified fragment containing SV40LTpolyA/IVS (described above). Following transformation and selection,
25 resulting plasmids are analyzed by restriction analysis using *Acl*I (3 fragments of 2994, 855 and 216 bp). A few are selected and additionally analyzed using *Hinc*II (2 fragments of 3466 bp and 599 bp, respectively), *Af*III (2 fragments of 2872 bp and 1193 bp, respectively) and *Bg*I (2 fragments of 2371 bp and 1694 bp, respectively).

30 A plasmid DNA showing all the expected fragments in the correct sizes is further checked by sequencing. A verified plasmid is designated as p2-dhfr-CDS.

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2. Construction of pEpo/dhfr

2.1 Preparation of pEpo/dhfr 21

p5 plasmid DNA is digested with *Bam*HI and *Eco*RI and the resulting 1792 bp fragment representing the cassette of SV40promoter-Eopgene-SV40terminator
5 is gel-purified.

Plasmid p2-dhfr-CDS is also digested with *Bam*HI and *Eco*RI and the linearized vector is gel purified and eluted using Supelco spin columns.

Additionally the DNA is dephosphorylated using alkaline phosphatase and purified with Amicon Micropure enzyme removers.

10 Both fragments, the 4053 bp p2-dhfr-CDS vector and the 1792 bp cassette from p5, are ligated (Ligation Express, Clontech) and transformed into *E.coli* SURE. Transformants colonies grown on LB medium supplemented with 70 mg/l ampicillin are hybridized using Epo gene (PCR-product) as a probe. Plasmid DNA is isolated from various positive clones and analyzed by digestion using restriction
15 endonuclease *Ncol*.

A clone showing the expected fragments (*Ncol*: 4085 bp and 1760 bp) is selected and designated as pEpo/dhfr-21. For additional purification the DNA is retransformed into *E.coli* SURE (see above) and plasmid DNA prepared using a "Midi-prep" procedure (Qiagen) from a culture inoculated by a single colony
20 (pEpo/dhfr-21-1).

Restriction analysis is performed using the following enzymes: *Bam*HI, *Hind*III, *Eco*RI, *Ncol*, *Not*I, *Pst*I, *Spe*I, *Sph*I, *Pvu*II, *Nar*I. All the expected fragments and sizes could be found, verifying the clone as a correct pEpo/dhfr-21.

2.2 The final construction of pEpo/dhfr

25 In the same way as for pEpo/neo the upstream region of the Epo gene in Epo/dhfr-21 is changed at position -3 referred to the start ATG. An additional nucleotide A is introduced to result in the purine base G at position -3 from start ATG. The Epo gene is reamplified as described in Example 1, section 4.2.

pEpo/dhfr-21 plasmid DNA is digested with *Ksp*I and *Not*I, to remove the
30 Epo gene. The 5259 bp fragment is then gel-purified.

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Both prepared DNAs are ligated to each other (Ligation Express, Clontech). Plasmid DNA from transformants is isolated and purified from colonies on LB medium supplemented with 70 mg/l ampicillin. DNAs are analyzed by restriction using *Ncol* in a first screening.

5 A positive clone is selected to isolate DNA using a "Midi prep" procedure (Qiagen). An extended restriction analysis is performed using *Bam*H, *Hind*III, *Eco*RI, *Ncol*, *Not*I, *Pst*I, *Spe*I, *Sph*I, *Pvu*II, *Nar*I. The expected fragments and sizes could be found, verifying the clone as a correct pEpo/dhfr.

Every single nucleotide of the whole cassette (SV40early promoter – dhfr
10 gene – SV40LTpolyA/IVS - SV40early promoter – Epo gene – SV40LTpolyA/IVS) inserted in the pBR322 vector-part is also confirmed by sequencing.

Example 2a - Construction of plasmids for recombinant expression of different proteins

15

In order to demonstrate the broad applicability of the system the following vector-host expression systems were constructed as shown in Table 5. The panel comprises expression of the human monoclonal antibodies 2F5 and 2G12 as subclass IgG1, IgM and IgA, the human monoclonal antibody 20 4E10 as subclass IgG1 and IgM and the human monoclonal antibody HB617 as subclass IgM, all expressed in CHO cells. Additionally, the human monoclonal antibody 4E10 IgG1 was expressed in Namalwa cells.

Example 3 - Recombinant CHO-cells generated using pEpo/neo and pEpo/dhfr

25

1-5x10⁴ cells per cm² are seeded in 25cm² T-flask bottles or 96-well plates the day before the lipofectin transfection is performed. The two plasmids are mixed at the ratio of 50:1 = Epo/neo:Epo/dhfr and allowed to adsorb to the lipofectin reagent (GIBCO/BRL) according to the manufacturer's protocol.

30

In brief, we used 0.25 µg DNA/cm² and 1.5 µl lipofectin-reagent/cm² and adjusted this DNA/lipid cocktail to 200 µl/cm² cell layer. Then the cells are overlaid with the transfection cocktail for four hours in serum-free DMEM, before the

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DNA-containing medium is replaced with cultivation medium. After cultivation for 24 hours in the serum-containing medium we switched to selection medium. Transfected cell-pools are first cultivated in selection medium to confluence and then in amplification medium (4.8×10^{-8} M MTX) before screening the cell culture 5 supernatants by ELISA for Epo production. Highest producers are determined, the MTX concentration increased two-fold and best producers used for further cultivation. 7 recombinant cell pools are selected and comparison made of the growing properties, the Epo productivity, the protein pattern (by western blot analysis), the Epo functionality and the chromosomal stability.

10 Transfection in T25-flasks is carried out with 2.5 μ g pEpo/neo, 0.05 μ g pEpo/dhfr and 15 μ l lipofectin (09/T25/1 and 09/T25/2) per T25-flask and with 2 μ g pEpo/neo, 0.4 μ g pEpo/dhfr and 15 μ l lipofectin (09/T25/3 and 09/T25/4) per T25-flask.

15 Additionally five plates are each transfected with 10 μ g pEpo/neo, 0.2 μ g pEpo/dhfr and 60 μ l lipofectin per plate (09/96/1 - 09/96/5), five plates with 8 μ g pEpo/neo, 1.6 μ g pEpo/dhfr and 60 μ l lipofectin per plate (09/96/6 - 09/96/10). Plates 11 and 12 are transfected with 6.25 μ g pEpo/neo, 0.08 μ g pEpo/dhfr and 37.5 μ l lipofectin each.

20 In brief, 0.25 μ g DNA/cm² and 1.5 μ l lipofectin-reagent/cm² are used and this DNA/lipid cocktail adjusted to 200 μ l/cm² cell layer.

The series of transfections is mainly done in microtitre plates since previous experiments show that the number of clones in one cultivation unit is maximum three to five. This means easier isolation of a monoclonal transfector than isolation from hundreds of clones in the T-flasks. Table 1 describes the number of 25 clones per 96-well plate and the ELISA titers with and without amplification pressure. Transfected cell pools are first cultivated in selection medium to confluence and then in amplification medium (4.8×10^{-8} M MTX) before screening the cell culture supernatants by ELISA for Epo production. Approximately 1000 growing wells are screened, and 50 such cultures tested for specific Epo-30 productivity with increased MTX concentration. Highest producers are determined, the MTX concentration increased two-fold and best producers used for further cultivation.

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The selection and first amplification steps are done in the 96-well plate and after screening all clones, growing in 4.8×10^{-8} M MTX, 7 clones are selected, designated 09/96/1F5, 09/96/3D5, 09/96/3H5, 09/96/5D4, 09/96/5H1, 09/96/6C5 and 09/96/7E6, and their growing properties, Epo productivity, protein pattern in western blots, Epo functionality tests and chromosomal stability compared.

The cell doubling time seems to be the same for all clones and they can be split 1:2 to 1:5 twice a week. Enhancing the MTX concentration from 9.6×10^{-8} M to 1.9×10^{-7} M also improves the productivity, while further doubling the MTX concentration does not influence the ELISA value. So subcloning is performed at 3.8×10^{-7} M MTX. Immunofluorescence is analyzed at 1.9×10^{-7} M MTX where the single cultures do not differ significantly.

Cell morphology is compared by light microscopy and Coulter Counter nucleus DNA analysis. Clones 09/96/7E9, 09/96/6C5, 09/96/5H1, 09/96/5D4 and 09/96/3D5 feature the same nucleus size distribution as the host cell line CHO-DHFR^r. In contrast, cell lines 09/96/1F5 and 09/96/3H5 have larger nuclei. It is known from previous experiments that this results from an extended number of chromosomes. It is therefore decided to use clone 09/96/3D5 for further stabilization.

The functionality test for Epo on TF-1 cells gives the same slope for all seven culture supernatants compared with the recombinant pharmaceutical product.

The recombinant protein is tested by SDS PAGE and western blotting at each MTX concentration and only minor changes are found in any of the recombinant culture supernatants. The clones produce Epo.

Summary and Discussion

The selection of a recombinant, Epo expressing CHO-cell line from the construction of eukaryotic expression vectors up to the transfection of mammalian cells and isolation of polyclonal Epo expressing cell pools is described. The analytical basis is set mainly with ELISA, immunofluorescence, western blotting and *in vitro* functionality tests. All these methods are established in concentration ranges that are capable of screening low producing cell pool culture

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supernatants with only ng/ml amounts as well as more stabilized recombinant cells.

A recombinant CHO-pool is generated, in which the gene copy number is amplified stepwise with up to 3.8×10^7 M MTX. These cells can be split 1:3 to 1:4
5 twice a week and each time elevated levels of Epo are detected in ELISA.

Example 3a - Recombinant CHO cells generated using expression plasmids described in Example 2a

10 The clone selection and gene amplifications, stabilization as well as adaptation to serum free cultivation conditions were performed as described in Examples 3, 4 and 5 disclosed herein. Examples of productivity of the generated cell lines are given in Table 6.

The expression rates depend primarily on the host cells used and on the POI,
15 independently of whether the polypeptide of interest was encoded by a single gene, or by two or three genes. Also the different viral promoters used have no significant influence, as shown.

Example 4 - Further selection of a recombinant cell-line

20

Recombinant cell-pool 09/96/3D5 is used for further stabilization. MTX concentration is increased stepwise to $0.38 \mu\text{M}$ MTX. At this amplification level recombinant 3D5 cells are subcloned with 10 and 20 cells per well. Screening of culture supernatants of wells with single clones is performed by ELISA. Table 2
25 shows the subcloning conditions and efficiencies of recombinant cell-pool 3D5 in the presence of $0.38 \mu\text{M}$ MTX. 300 supernatants of single clones are tested. Clones that have elevated Epo titers four days after passaging are selected with $0.77 \mu\text{M}$ MTX in 24 well plates.

Seven of these clones are conserved in liquid nitrogen and selected for
30 further amplification of gene copy number by increasing MTX concentration to $1.54 \mu\text{M}$.

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Table 3 compiles the plating conditions and efficiencies of the second round of stabilization. Here the clones 09/96/3D5/1H9 and 09/96/3D5/18E5, are subcloned a final time with 1.54 µM MTX. The cell counts per well are reduced to 4 cells. 260 single clones are screened of which more than twenty clones of 5 each subcultivation are transferred to T-flasks and screened for specific productivity. The final production clones are settled by criteria such as specific expression rate, growth conditions and nucleus size distribution. Clones showing tetraploidy are discarded because of the experience that such cells tend to show complicated growth patterns in bioreactors. After screening the following six 10 subclones (four 1H9 and two 18E5 subclones) are chosen, which are frozen in liquid nitrogen.

09/96/3D5/1H9/4C2	09/96/3D5/1H9/6C2	09/96/3D5/1H9/6D4
09/96/3D5/1H9/15B4	09/96/3D5/18E5/7A6	09/96/3D5/18E5/15C3

15

Example 5 - Adaptation to serum-free cultivation medium

The final six recombinant cell-lines from Example 4 are chosen for adaptation to serum-free cultivation conditions after the last subcloning step.

20 Cells are seeded in the 7. – 12. passage after subcloning with approximately 5×10^4 cells/cm² into T25-flasks and are cultivated 3-4 days to confluence. At this time point the medium is replaced completely with serum-free adaptation medium and afterwards 80% of the medium is renewed daily. All suspended cells are returned to the culture. After the adaptation time, when 25 nearly all cells grew in suspension, the clones are passaged twice a week and cultivated as suspension-culture.

Clones are cultivated for 11 – 13 passages in serum-free adaptation medium before cryopreservation. Six ampoules with 5×10^6 cells each are frozen of every cell-line in liquid nitrogen with serum-free freezing medium. After thawing, 30 the clones are cultivated in serum-free production medium. Analytical characterization to select for the production clone is done with supernatants in the second or third passage after thawing.

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Analytical tests included:

Specific growth rate $[\mu]$ - ($\mu = \ln(X_2/X_1) / \text{days}$)

Specific productivity $[q_p]$ - ($Q_p = \text{product-generation} \times 10^6 / (\text{cell counts} \times \text{days})$)

Western blot

5 Isoelectric focussing

DNA content and stability

Clone stability

All six cell-lines could be grown in serum-free growth media and are split twice a week. Cryopreservation is also performed without serum and after

10 thawing the cultivation medium is switched to serum free production medium.

This formulation is enriched in glucose and amino acids.

After 5 passages different protein and cellular parameters are determined and one production clone (09/96/3D5/1H9/6C2; abbreviated 6C2) and one back up clone (09/96/3D5/1H9/4C2; abbreviated 4C2) selected.

15

Example 5a – Adaptation of CHO K1 to serum-free and protein-free cultivation conditions

CHO K1 cells were cultivated with medium supplemented with 10% FCS
20 and seeded 4 days prior to adaptation into a T25 flask containing 10 ml of cultivation medium with FCS. At this time point adaptation starts with an 80% replacement of medium against the serum-free medium. Afterwards 80% of the medium was renewed every third day and later on, 80% of the medium was renewed daily.

25 After 10-14 of such renewals of 80% medium nearly all cells grew in suspension. The suspension cells were centrifuged for 10 minutes at 110g and re-suspended in serum free medium.

After this procedure, the cells were cultivated by passaging them twice a week (see Figure 1).

30

Medium:

DMEM/HAMs 1:1, protein-free-supplement, Lutrol®, L-glutamine, soya peptone.

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Example 5b – Adaptation of CHO K1 to serum-free and protein-free cultivation conditions without plant-derived hydrolysate

- One ampoule of the cells of example 5a has been reconstituted using the serum-free medium. The cells have been grown in T25 flasks. After some passages 80% of the medium was replaced each day by a medium without soya digest. After one month of adaptation the cells were centrifuged for 10 minutes at 170g. The cells were re-suspended in the protein free medium and cultivated in a spinner flask over some weeks.
- 10 Medium: DMEM/HAMs 1:1, protein-free supplement, Lutrol®, L-glutamine (see Figure 2)

Example 5c – Adaptation of a recombinant SP2/0 cell line expressing a monoclonal antibody to serum-free and protein-free cultivation conditions

- 15 One ampoule of recombinant SP2/0 cells has been reconstituted from a commercial available serum free medium (without knowledge of medium composition) to our protein free medium. The cells have been grown in T25 flasks. After this procedure the cells have been cultivated by passaging them twice a week in spinner vessels (125ml, 80ml filling volume).
- 20 Medium: RPMI/DMEM/HAMs 2:2:1, protein-free supplement, Lutrol®, L-glutamine (see Figure 3).

Example 6 - Comparison of the recombinant cell-lines

- 25 Growth properties of the six cell-lines from Examples 4 and 5 are calculated over several weeks by determining the cell counts in culture as well as splitting ratios during passaging. The Epo productivity is tested by ELISA. From that data the specific productivity and specific growth rate as described above are
- 30 calculated.

Table 4 summarizes the data received under standard cultivation conditions with a splitting ratio of 1:3 after three days cultivation.

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Cell counts (measured by Coulter Counter) after splitting and after additional three days are shown.

SDS-PAGE under reducing conditions

The supernatants of the six cell-lines are separated by SDS-PAGE and compared
5 for differences in the molecular weight. The six supernatants indicate identical SDS patterns with a smear, commonly seen in such highly glycosylated proteins (data not shown).

The comparable commercial available product migrates as a more distinct band probably arising from separating distinct bands during down stream
10 processing.

IEF-Western blot

The IEF-western blot analysis should reflect potential microheterogeneities of the glycoproteins. According to the amount of protein that is loaded on the gel to fourteen bands become visible. There is one characteristic double-band seen on
15 the western blot approximately in the middle of the gel; the next band down under this double-band is defined as band number one and 9 to 10 bands are visible in this acidic part of the gel. The comparable commercial product gave four major bands that correspond to band number six to nine in the heterogeneous product.

20 DNA content of recombinant cells

The DNA content is proportional to the numbers of chromosomes of cell-lines. The stability of a recombinant cell-line is in part influenced by the chromosomal count and the identity of DNA content is verified by comparison to the host cell-line (CHO dhfr).

25 Summary and Discussion

The isolation of recombinant, Epo expressing CHO cell-lines is described herein. After two rounds of subcloning six cell-lines are compared for different properties as the basis for the designation of one final production clone. The analytical basis is mainly ELISA, western blotting and IEF tests as well as DNA measurement by
30 FACS analysis.

The western blot pattern of the recombinant culture supernatants shows several additional lower molecular weight bands compared to the commercial

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purified protein. One explanation is that these additional bands represent isoforms which are removed during the down stream processing leading to the commercial product compared. Another possibility that artificial bands are being detected due to incomplete uptake of SDS.

- 5 Isoelectric focussing gives identical isoform-distribution for all cell culture supernatants, irrespective of their Qp.

The best producing clone and easiest-handling is clone 6C2 which is chosen as production clone. As back-up clone 4C2 is chosen. Both clones can be propagated in roller bottles.

10 **Example 7 - Cultivation of CHO cells in T-Flasks**

Recombinant human Erythropoietin is produced in a Chinese hamster ovary cell line (CHO) under serum free conditions in T-Flasks. The culture is seeded with a 2.67×10^5 cells/mL. After a three day incubation period a final cell density of

15 9.35×10^5 cells/mL is reached ($= > \mu = 0.42 \text{ days}^{-1}$).

Examples 1 to 6 describe the preparation of a number of CHO clones which express Epo. Of the six clones obtained in Examples 4 and 5, clone CHO 6C2 is chosen due to its superior high cell specific productivity and its high specific growth rate.

20

Example 8 - Cultivation of CHO cells in a bioreactor

The CHO cell line 6C2 is cultivated in Fed-Batch (T43C6C2) mode in a 150 L bioreactor. Using a cell culture medium consisting of amino acid-supplemented 50:50 DMEM/Hams F12 and containing 0.25% of a plant peptide, 0.1% Lutrol®,

25 1.54 μM methotrexate (MTX), 4 g/L glucose, 2.5 g/L NaHCO₃, ethanolamine, ferric citrate, ascorbic acid and sodium selenite. The medium did not contain any expensive functional proteins (recombinant or from natural sources). Components derived from an animal origin are present. The cells are seeded at around 5×10^5 cells/mL in 56 L medium. The pO₂ is set to 50% air saturation, temperature to
30 37°C and the pH to 7.0 and kept constant during the course of the fermentation.

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The glucose concentration is kept above 1g/L. After 4 days the reactor is filled to 150 L with fresh medium. After day 9 the batch is extended by adding 1875 mL of a nutrient concentrate containing amino acids, a carbohydrate and a plant derived peptone. After 10 days another 1875 mL of the nutrient concentrate 5 are added. Two days later (day 12) the supernatant containing erythropoietin is harvested.

Example 9 - Production of erythropoietin in a bioreactor without methotrexate

The CHO cell line 6C2 is cultivated in Fed-Batch (Kamp 4 B5-1 and 2) mode in a 5-L bioreactor. The medium is as in Example 9. The first bioreactor (Kamp 4 10 B5-1) is set up with 1.54 μ m MTX in the medium, and the second (Kamp 4 B5-2) without MTX.

The glucose concentration is kept above 1g/L. The cells are seed at around 5 \times 10⁵ cells/mL in 1250-mL medium. The pO₂ is set to 50% air saturation, temperature to 37°C and the pH to 7.0 and kept constant during the course of 15 the fermentation. After 2 days the reactor is filled to 5 L with fresh medium. After day 6, 7, 8, 9 and 10 the batch is extended by adding 50 to 122-mL of a nutrient concentrate containing amino acids, a carbohydrate and a plant derived peptone. On day 11 the supernatant containing erythropoietin is harvested.

The cultivation without methotrexate is found to be superior due to the 20 better glycosylation pattern.

Example 10 - Production of Erythropoietin in a bioreactor with enriched medium

The CHO cell line 6C2 is cultivated in Fed-Batch (Kamp 11 B5-1 and 2) mode in a 5 L bioreactor. Bioreactor 1 is operated as in Example 10 (Kamp 4 B5-2). In bioreactor 2 a cell culture medium is used consisting of an enriched amino 25 acid supplemented 50:50 DMEM/Hams F12 and containing a 0.325% of a plant peptide, 0.1% Lutrol®, 6.4 g/L glucose, 2.5 g/L NaHCO₃, ethanolamine, ferric-citrate, ascorbic acid and sodium selenite and 0.6 g/L phosphate. The cells are seed at around 5 \times 10⁵ cells/mL in 1250 mL medium. The pO₂ is set to 50% air saturation, temperature to 37°C. The pH is set to 7.1 at the beginning. During the 30 course of the fermentation it is reduced step wise to 6.9.

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Over the course of the cultivation the glucose concentration in bioreactor 2 is kept between 3 to 4 g/L. After 2.5 days the reactor is filled to 5 L with fresh medium. After day 6, 7, 8, 9 and 10 the batch is extended by adding an enriched nutrient concentrate containing amino acids, a carbohydrate and a plant derived 5 peptone. On day 11 the supernatant containing Epo is harvested.

The cultivation with a nutrient enriched medium (amino acid, glucose, plant peptone and phosphate) as well as the pH-shift from 7.1 to 6.9 is found to more than double the final Epo concentration at a comparable glycosylation profile.

10 **Example 11 - Production of erythropoietin in a bioreactor lacking components derived from animals**

The CHO cell line 6C2 is cultivated in Fed-Batch (Kamp 17 B5-1 and 3) mode in a 5-L bioreactor. All parameters are set as in Example 10 (Kamp 4 B5-2) if not otherwise noted. In bioreactor 2, a cell culture medium is used which does 15 not contain any components derived from animals. For example the amino acid tyrosine or cysteine, which are typically derived from an animal (like salmon or human hair) have been replaced by synthetic amino acids.

After 2.5 days the reactor is filled to 5 L with fresh medium. After day 5, 6, 7, 8 and 9 the batch is extended by adding a nutrient concentrate containing 20 amino acids, a carbohydrate and a plant derived peptone. On day 9 to 10 the supernatant containing erythropoietin is harvested.

A medium not containing any components of animal origin is found to yield a comparable final Epo concentration. However the culture grows slower and needs an additional nutrient concentrate addition.

25

Example 12 - Production of Erythropoietin in a bioreactor with enriched medium (vitamins, trace elements)

The CHO cell line 6C2 is cultivated in Fed-Batch (Kamp 12 C) mode in a 10 L bioreactor. The bioreactor is operated as in Example 11 (Kamp 11 B5-2) with 30 the following exceptions:

A cell culture medium is used consisting of an enriched amino acid supplemented 50:50 DMEM/Hams F12 and containing a 0.325% of a plant

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peptide, 0.1% Lutrol®, 6.4 g/L glucose, 2.5 g/L NaHCO₃, ethanolamine, ferric-citrate, vitamins, trace elements and sodium selenite and 0.6 g/L phosphate. The content in the concentrate is doubled and enriched with vitamins.

The cells are seeded at around 5x10⁵ cells/mL in 4500 mL medium. The 5 pO₂ is set to 50% air saturation, temperature to 37 °C. The pH is set to 7.1 at the beginning. During the course of the fermentation it is reduced step wise to 6.9.

Over the course of the cultivation the glucose concentration in the bioreactor is kept between 3 to 4 g/L. After 3 days the reactor is filled to 10 L with fresh medium. After day 6, 7, 8, 9, 10, 11 and 12 the batch is extended by 10 adding the enriched nutrient concentrate. On day 13 the supernatant containing erythropoietin is harvested.

Example 12a - Cultivation of a recombinant human monoclonal antibody IgG₁ in a fed-batch system

15 The recombinant CHO cell line was cultivated in fed-batch mode in a 8 L bioreactor. Using a cell culture medium consisting of amino acid-supplemented 50:50 DMEM/Ham's F12, 0.1% Lutrol®, 1 µM methotrexate (MTX), 4 g/L glucose, 2.5 g/L NaHCO₃, ethanolamine, ferric citrate, ascorbic acid and sodium selenite. Feeding was carried out continuously by addition of a stoichiometric (4x) medium 20 concentrate. The medium did not contain any functional proteins (recombinant or from natural sources) or functional parts thereof, nor any animal or plant-derived extracts or hydrolysates (peptones). The cells were seeded at around 5x10⁵ cells/mL in 2 L medium. The pO₂ was set to 50% air saturation, the temperature to 37 °C and the pH to 7.0 and kept constant during the course of the 25 fermentation (see Figure 4).

Example 12b - Cultivation of a recombinant human monoclonal antibody IgG₁ in a continuous perfused fluidized bed reactor

The recombinant CHO cell line was cultivated in continuous mode in a 30 continuous perfused 2 L fluidized bed reactor using macroporous microcarriers to which the cells adhered. The cell culture medium consisted of amino acid-supplemented 50:50 DMEM/Ham's F12, 0.1% Lutrol®, 1 µM methotrexate (MTX),

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4 g/L glucose, 2.5 g/L NaHCO₃, ethanolamine, ferric citrate, ascorbic acid and sodium selenite. The medium did not contain any functional proteins (recombinant or from natural sources) or functional parts thereof, nor any animal or plant-derived extracts or hydrolysates (peptones). The pO₂ is set to 50% air saturation, 5 temperature to 37°C and the pH to 7.0 and kept constant during the course of the fermentation. (see Figure 5)

Example 12c - Cultivation of a recombinant human monoclonal antibody IgM in a fed-batch system

10 The recombinant CHO cell line was cultivated in fed-batch mode in a 5 L bioreactor, using a cell culture medium consisting of amino acid-supplemented 50:50 DMEM/Ham's F12, 0.1% Lutrol®, 1 µM methotrexate (MTX), 4 g/L glucose, 2.5 g/L NaHCO₃, ethanolamine, ferric citrate, ascorbic acid and sodium selenite. Feeding was carried out discontinuously by addition of a nutrient concentrate. The 15 medium did not contain any functional proteins (recombinant or from natural sources) or functional parts thereof, nor any animal or plant-derived extracts or hydrolysates (peptones). The cells are seeded at around 5x10⁵ cells/mL in 2 L medium. The pO₂ is set to 50% air saturation, temperature to 37°C and the pH to 7.0 and kept constant during the course of the fermentation. (see Figure 6)

20

Example 12d - Cultivation of a recombinant human monoclonal antibody IgM in a stirred tank reactor equipped with an ultrasonic cell retention device

The recombinant CHO cell line is cultivated in continuous mode in a continuous perfused 5 L stirred tank reactor equipped with an ultrasonic cell 25 retention device. Using a cell culture medium consisting of amino acid-supplemented 50:50 DMEM/Hams F12 and containing 0.25% of a plant peptide, 0.1% Lutrol®, 1 µM methotrexate (MTX), 4 g/L glucose, 2.5 g/L NaHCO₃, ethanolamine, ferric citrate, ascorbic acid and sodium selenite. The medium did not contain any functional proteins (recombinant or from natural sources) or 30 functional parts thereof, nor any animal or plant-derived extracts or hydrolysates (peptones). The pO₂ was set to 50% air saturation, temperature to 37°C and the pH to 7.0 and kept constant during the course of the fermentation. (see Figure 7)

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Example 12e - Cultivation of a murine hybridoma cell line in batch mode to a scale of 500 L

The murine hybridoma cell line was cultivated in batch mode to a scale volume of 500 L. The cultivation system consisted of two stirred tank reactors 5 (15 and 200 L respectively) and an airlift reactor (500 L). For cell cultivation a RDH-medium containing 0.05 % Lutrol®, 2.5 g/l NaHCO₃, 8 mMol L-glutamine, ethanolamine, ferric citrate, ascorbic acid and sodium selenite was used. The fermentation was carried out at setpoint 37 °C, pH 7.0 and DOC of 30 % air saturation an kept constant via an industrial process control unit (direct digital 10 control). (see Figure 8)

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Table 1: recombinant CHO-cells: transfections with Epo/neo and Epo/dhfr

T25 transfections			
transfection	number of clones per T25	Qp, 9.6×10^{-8} M MTX [μg/10^6cells/d]	
09/T25/1 (50:1)	136	0.16	
09/T25/2 (50:1)	107	2.6	
09/T25/3 (5:1)	459	0.14	
09/T25/4 (5:1)	648	0.9	
96-well transfections			
transfection	number of clones per plate	selected clone	
09/96/1 (50:1)	47	1F5	
09/96/2 (50:1)	46		
09/96/3 (50:1)	50	5D5, 3H5	
09/96/4 (50:1)	52		
09/96/5 (50:1)	49	5D4, 5H4	
09/96/6 (5:1)	416	6C5	
09/96/7 (5:1)	556	7E9	
09/96/8 (5:1)	392		
09/96/9 (5:1)	427		
09/96/10 (5:1)	352		
09/96/11 (75:1)	49		
09/96/12 (75:1)	60	12A9	
Amplification of different clones			
clone	Qp [μg/10^6cells/d]		
	9.6×10^{-8} M MTX	1.9×10^{-7} M MTX	3.8×10^{-7} M MTX
09/96/1F5	11	11.4	17.1
09/96/3D5	8.4	14.4	11.4
09/96/3H5	8.2	14.1	12.9
09/96/5D4	4.9	3.8	3.6
09/96/5H1	4.7	7.1	6.7
09/96/6C5	4.2	3.8	3.6
09/96/7E9	5.5	8.8	8.9
09/96/12A9	6		

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Table 2: Subcloning conditions and plating efficiencies of cell-pool 3D5 with 0.38 μM MTX

Number of 96 well plates	Number of seeded cells/well	% growing wells	% single clones
25 (no. 6-30)	10	13%	77%
5 (no. 1-5)	20	24%	54%

Table 3: Subcloning conditions and plating efficiencies of Subclone 3D5/1H9 and 3D5/18E5 with 1.54 μM MTX

3D5/1H9

Number of 96 well plates	Number of seeded cells/well	% growing wells	% single clones
8 (no. 9-16)	10	6.4%	85%
8 (no. 1-8)	30	19%	46%

3D5/18E5

Number of 96 well plates	Number of seeded cells/well	% growing wells	% single clones
8 (no. 9-16)	4	15%	56%
8 (no. 1-8)	8	29%	44%

Table 4: Specific productivity and growth properties of recombinant CHO-clones

	4C2	6C2	6D4	15B4	7A6	15C3
Inoculated cell number [$\times 10^5$ cells/ml]	2.17	2.67	2.89	0.71	2.38	2.16
Final cell number [$\times 10^5$ cells/ml]	7.27	9.35	8.8	2.61	6.41	6.15
Specific growth rate μ [days ⁻¹]	0.4	0.42	0.37	0.43	0.33	0.35

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Table 5: Examples of vector/host-constructs for recombinant expression of different isoforms of human recombinant monoclonal antibodies and recombinant hGH under various promoters

Recombinant protein/(host)	Promoter/translation-initiation-site			Selektion-marker	Amplification-marker
	POI 1	POI 2	POI 3		
2F5 IgG (CHO)	HC/RSV-LTR (pRC/RSV) tcc <u>atg gag</u>	LC/RSV-LTR (pRC/RSV) tcc <u>atg gac</u>	-	Neo®, SV40 E pr. <u>cgc atg att</u>	dhfr SV40 E pr. tga <u>atg gtt</u>
2G12 IgG (CHO)	HC/RSV-LTR (pRC/RSV) tcc <u>atg gag</u>	LC/RSV-LTR (pRC/RSV) tcc <u>atg gac</u>	-	Neo®, SV40 E pr. <u>cgc atg att gaa</u>	dhfr SV40 E pr. tga <u>atg gtt</u>
4E10 IgG (CHO)	HC/CMV IE pr. (pCI neo) ccc <u>atg gag</u>	LC/CMV IE pr. (pCI neo) ccc <u>atg gac</u>	-	Neo®, SV40 E pr. <u>acc atg att</u>	dhfr SV40 E pr. ctt <u>atg gtt</u>
2F5 IgM (CHO)	HC/SV40 E pr. (pIRES neoSV40) gcc <u>atg gag</u>	LC/SV40 E pr. (pIRES neoSV40) gcc <u>atg gac</u>	j-chain/SV40 E pr. (pIRES neoSV40) ggg <u>atg aag</u>	-	j-chain/SV40 E pr. (pIRES neoSV40) tgg <u>atg gtt</u>
2G12 IgM (CHO)	HC/SV40 E pr. (pIRES neoSV40) gcc <u>atg gag</u>	LC/SV40 E pr. (pIRES neoSV40) gcc <u>atg gac</u>	j-chain/SV40 E pr. (pIRES neoSV40) ggg <u>atg aag</u>	-	j-chain/SV40 E pr. (pIRES neoSV40) tgg <u>atg gtt</u>
4E10 IgM (CHO)	HC/SV40 E pr. (pIRES neoSV40) gcc <u>atg gag</u>	LC/SV40 E pr. (pIRES neoSV40) gcc <u>atg gac</u>	j-chain/SV40 E pr. (pIRES neoSV40) ggg <u>atg aag</u>	-	j-chain/SV40 E pr. (pIRES neoSV40) tgg <u>atg gtt</u>
2F5 IgA (CHO)	HC/SV40 Promotor (pIRES neoSV40) gcc <u>atg gag</u>	LC/SV40 E pr. (pIRES neoSV40) gcc <u>atg gac</u>	j-chain/SV40 E pr. (pIRES neoSV40) ggg <u>atg aag</u>	-	j-chain/SV40 E pr. (pIRES neoSV40) tgg <u>atg gtt</u>

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Table 5 continued

2G12 IgA (CHO)	HC/SV40 E pr. (pIRES_neoSV40) gcc <u>atg</u> gag	LC/SV40 E pr. (pIRES_neoSV40) gcc <u>atg</u> gac	j-chain/SV40 E pr. (pIRES_neoSV40) ggg <u>atg</u> aag	-	j-chain/SV40 E pr. (pIRES_neoSV40) tgg <u>atg</u> gtt
4E10 IgG (Namalwa)	HC/CMV IE pr. (pCI_neo) <u>ccc</u> <u>atg</u> gag	LC/CMV IE pr. (pCI_neo) <u>ccc</u> <u>atg</u> gac	-	Neo®, SV40 E pr. acc <u>atg</u> att	dhfr mut SV40 E pr. ctt <u>atg</u> gtt
HB 617 IgM (CHO)	HC/CMV IE pr. (pIRES dhfr) <u>acc</u> <u>atg</u> gac	LC/CMV IE pr. (pIRES A/B) <u>aag</u> <u>atg</u> gtg	j-chain/CMVIE pr. (pIRES A/B) ggg <u>atg</u> aag	Neo®, SV40 E pr. acc <u>atg</u> att	dhfr/CMV IE pr. (pIRES dhfr) tgg <u>atg</u> gtt
hGH (CHO)	hGH/SV40 E pr. acc atg gct	-	-	-	dhfr SV40 E pr. ctt <u>atg</u> gtt

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Table 6: Specific productivity of recombinant Immunoglobulins and
recombinant hGH expressed in different host cells

Recombinant protein/ (host)	Spec. productivity in T- flask [pg/cell*day]	Spec. productivity in pilot scale fermentation [pg/cell*day]
2F5 IgG (CHO)	9	9
2G12 IgG (CHO)	10	9
4E10 IgG (CHO)	15	15
2F5 IgM (CHO)	60	
2G12 IgM (CHO)	15	
4E10 IgM (CHO)	68,2	
2F5 IgA (CHO)	10	
2G12 IgA (CHO)	1	
4E10 IgG (Namalwa)	3,7	
HB 617 IgM (CHO)	58,1	55

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All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are apparent to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.

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CLAIMS

We claim:

1. A method for producing a polypeptide of interest which method comprises providing a hybridoma which comprises a nucleotide sequence encoding a polypeptide of interest or a transformed host cell which comprises a nucleotide sequence which encodes a polypeptide of interest and which directs expression of the polypeptide of interest in the transformed host cell; providing a culture medium suitable for mammalian cell culture; and culturing the hybridoma or transformed host cell in the culture medium under conditions that allow for expression of the polypeptide of interest, characterized in that the culture medium
 - (i) comprises water, a buffer, an energy source, amino acids, a lipid source or precursor, a source of iron, non-ferrous metal ions, inorganic salts, vitamins and cofactors; and
 - (ii) is free from each of a plant-derived or animal-derived peptone, a functional protein, and a full-length polypeptide.
2. A method according to claim 1, wherein the culture medium comprises a protein-free supplement comprising a source of iron, preferably ferric citrate; a source of selenium, preferably sodium selenite; ascorbic acid, and ethanolamine.
3. A method according to claim 1 or 2, wherein the culture medium comprises a shear force protective agent, preferably selected from the group consisting of polyalkylene glycols, preferably polyethylene glycols and copolymers of ethylene oxide and propylene oxide.
4. A method according to any one of claims 1 to 3, wherein the hybridoma or transformed host cell is adherent to a solid carrier, preferably a microcarrier bead, and the culture medium is free from a ferric citrate.
5. A method according to any one of claims 1 to 4, further comprising a step of freezing and/or maintaining the hybridoma or transformed host cell, or any

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mammalian cell from which the hybridoma or transformed host cell is derived, prior to or after any cell culturing step.

6. A method according to claim 5, wherein said step of freezing and/or maintaining is carried out in a serum-free and protein-free medium.

7. A method according to claim 6, wherein the medium for freezing and/or maintaining consists of water, a buffer, preferably PBS, and at least one cryoprotective agent, preferably selected from the group consisting of dimethylsulfoxide (DMSO) and polyvinylpyrrolidone (PVP).

8. A method according to claim 7, wherein the medium for freezing and/or maintaining consists of water, PBS, and 2 – 8 g/100ml, preferably 5 g/100ml, of DMSO, and 15 – 25 g/100ml, preferably 20 g/100ml, of PVP.

9. A method according to any one of claims 1 to 8, wherein the transformed host cell comprises

(a) a first polynucleotide vector comprising a first nucleotide sequence that encodes a polypeptide of interest, and, optionally, a second nucleotide sequence that encodes a selectable marker; and a second polynucleotide vector comprising a third nucleotide sequence that encodes a different selectable marker and that is free from a nucleotide sequence encoding a polypeptide of interest; or, alternatively,

(b) a polynucleotide vector comprising a first nucleotide sequence that encodes a polypeptide of interest, and, optionally, a second nucleotide sequence that encodes a selectable marker, and further a third nucleotide sequence that encodes a different selectable marker; and, optionally,

(c) at least one more polynucleotide vector comprising a nucleotide sequence encoding another polypeptide of interest and, optionally, a nucleotide sequence encoding a selectable marker;

the polynucleotide vectors being integrated into the genome of the transformed host cell.

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10. A method according to claim 9, wherein said third nucleotide sequence encodes dihydrofolate reductase (dhfr).
11. A method according to claim 10, wherein the transformed host cell is cultured in the presence of methotrexate (MTX).
12. A method according to any one of claims 9 to 11, wherein said second nucleotide sequence of the first or of at least one polynucleotide vector encodes resistance to an antibiotic.
13. A method according to claim 12, wherein the transformed host cell is cultured in the presence of said antibiotic.
14. A method according to any one of claims 9 to 11, wherein the transformed host cell does not contain a nucleotide sequence encoding resistance to an antibiotic.
15. A method according to any one of claims 9 to 14, wherein the transformed host cell is cultured in the absence of an antibiotic.
16. A method according to any one of claims 1 to 15, wherein the polypeptide of interest is selected from the group consisting of human growth hormone, human monoclonal antibodies, preferably of subclasses IgG, IgM, and IgA, and monoclonal human/mouse chimeric antibodies.
17. A method according to any one of claims 9 to 16, wherein the transformed host cell comprises at least two polynucleotide vectors that encode different polypeptides of interest and wherein said different polypeptides of interest are different parts of an antibody, particularly the light and heavy chains of an antibody.

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18. A method according to any one of claims 1 to 17, wherein the hybridoma or transformed host cell is obtained from a mammalian cell selected from the group consisting of rodent cells, particularly mouse hybridomas, human/mouse heterohybridomas, SP2-0 cells, NS0 cells, baby hamster kidney cells, CHO cells, CHO dhfr⁻ cells, CHO-K1 cells, and recombinant cells obtained from any one of these cell lines.

19. A cell culture medium suitable for culturing mammalian cells, characterized in that it

(i) comprises water, a buffer, an energy source, amino acids, a lipid source or precursor, a source of iron, non-ferrous metal ions, inorganic salts, vitamins and cofactors; and

(ii) is free from each of a plant-derived or animal-derived peptone, a functional protein, and a full-length polypeptide.

20. The medium according to claim 19, which comprises a protein-free supplement comprising a source of iron, preferably ferric citrate; a source of selenium, preferably sodium selenite; ascorbic acid, and ethanolamine.

21. The medium according to claim 19 or 20, which comprises a shear force protective agent, preferably selected from the group consisting of polyalkylene glycols, preferably polyethylene glycols and copolymers of ethylene oxide and propylene oxide.

22. The medium according to claim 19, which is suitable for culturing mammalian cells adherent to a solid carrier, preferably a microcarrier, and which is free from a source of iron.

23. The medium according to claim 19, which is suitable as a selection medium, and which comprises methotrexate, and, optionally, an antibiotic.

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24. The medium according to claim 19, which comprises DMEM, Ham's F12, L-glutamine, protein-free supplement, a polyalkylene glycol, methotrexat (MTX), and, optionally, G418.
25. The medium according to claim 19, which comprises DMEM/Ham's F12 in a ratio of 1:1, supplemented with 2-8mM, preferably 6mM, L-glutamine, 1x protein-free supplement, 0.05-0.3%, preferably 0.1%, of a polyalkylene glycol as a shear force protective agent, 0.02×10^{-6} M - 10×10^{-6} M, preferably, 1.54×10^{-6} M, of methotrexat (MTX), and, optionally, 0.1-1 mg/ml, preferably 0.5 mg/ml G418.
26. The medium according to any one of claims 19 to 25, which comprises glucose, preferably at a concentration of 0.5-10 g/l, particularly 1-4 g/l, and NaHCO₃, preferably at a concentration of 0.05-5 g/l, particularly 2.5 g/l.
27. A medium suitable for freezing and/or maintaining a mammalian cell, characterized in that it consists of water, a buffer, preferably PBS, and at least one cryoprotective agent, preferably selected from the group consisting of dimethylsulfoxide (DMSO) and polyvinylpyrrolidone (PVP).
28. The medium according to claim 27, which comprises 2 – 8 g/100ml, preferably 5 g/100ml, of DMSO, and 15 – 25 g/100ml, preferably 20 g/100ml, of PVP.
29. Use of a medium as defined in any one of claims 19 to 28 in a method for producing a polypeptide of interest involving a hybridoma or transformed eukaryotic host cell expressing said polypeptide of interest.
30. Use according to claim 29, wherein the polypeptide of interest is selected from the group consisting of human growth hormone, human monoclonal antibodies, preferably of subclasses IgG, IgM, and IgA, and monoclonal human/mouse chimeric antibodies.

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31. Use according to claim 29 or 30, wherein the hybridoma or transformed host cell is obtained from a mammalian cell selected from the group consisting of rodent cells cells, particularly mouse hybridomas, human/mouse heterohybridomas, SP2-O cells, NSO cells, baby hamster kidney cells, CHO cells, CHO dfr⁻ cells, CHO-K1 cells, and recombinant cells obtained from any one of these cell lines.

32. A transformed host cell that comprises

- (a) a first polynucleotide vector comprising a first nucleotide sequence that encodes a polypeptide of interest, and, optionally, a second nucleotide sequence that encodes a selectable marker; and a second polynucleotide vector comprising a third nucleotide sequence that encodes a different selectable marker and that is free from a nucleotide sequence encoding a polypeptide of interest; or, alternatively,
 - (b) a polynucleotide vector comprising a first nucleotide sequence that encodes a polypeptide of interest, and, optionally, a second nucleotide sequence that encodes a selectable marker, and further a third nucleotide sequence that encodes a different selectable marker; and, optionally,
 - (c) at least one more polynucleotide vector comprising a nucleotide sequence encoding another polypeptide of interest and, optionally, a nucleotide sequence encoding a selectable marker;
- the polynucleotide vectors being integrated into the genome of the transformed host cell.

33. A transformed host cell according to claim 32, wherein said third nucleotide sequence encodes dihydrofolate reductase (dhfr).

34. A transformed host cell according to claim 32 or 33, wherein said second nucleotide sequence of the first or of at least one polynucleotide vector encodes resistance to an antibiotic.

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35. A transformed host cell according to claim 32, which does not contain a nucleotide sequence encoding resistance to an antibiotic.
36. A transformed host cell according to any one of claims 32 to 35, wherein the polypeptide of interest is selected from the group consisting of human growth hormone, human monoclonal antibodies, preferably of subclasses IgG, IgM, and IgA, and monoclonal human/mouse chimeric antibodies.
37. A transformed host cell according to any one of claims 32 to 36, which is obtained from a mammalian cell selected from the group consisting of rodent cells, particularly mouse hybridomas, human/mouse heterohybridomas, SP2-0 cells, NS0 cells, baby hamster kidney cells, CHO cells, CHO dfr⁻ cells, CHO-K1 cells, and recombinant cells obtained from any one of these cell lines.
38. Use of a hybridoma or of a transformed host cell defined in any one of claims 32 to 37, in a method for producing a polypeptide of interest as defined in any one of claims 1 to 18.
39. A composition comprising a polypeptide of interest obtained in a method as defined in any one of claims 1 to 18, the polypeptide of interest preferably being selected from the group consisting of human growth hormone, human monoclonal antibodies, preferably of subclasses IgG, IgM, and IgA, and monoclonal human/mouse chimeric antibodies.

- 1/4 -

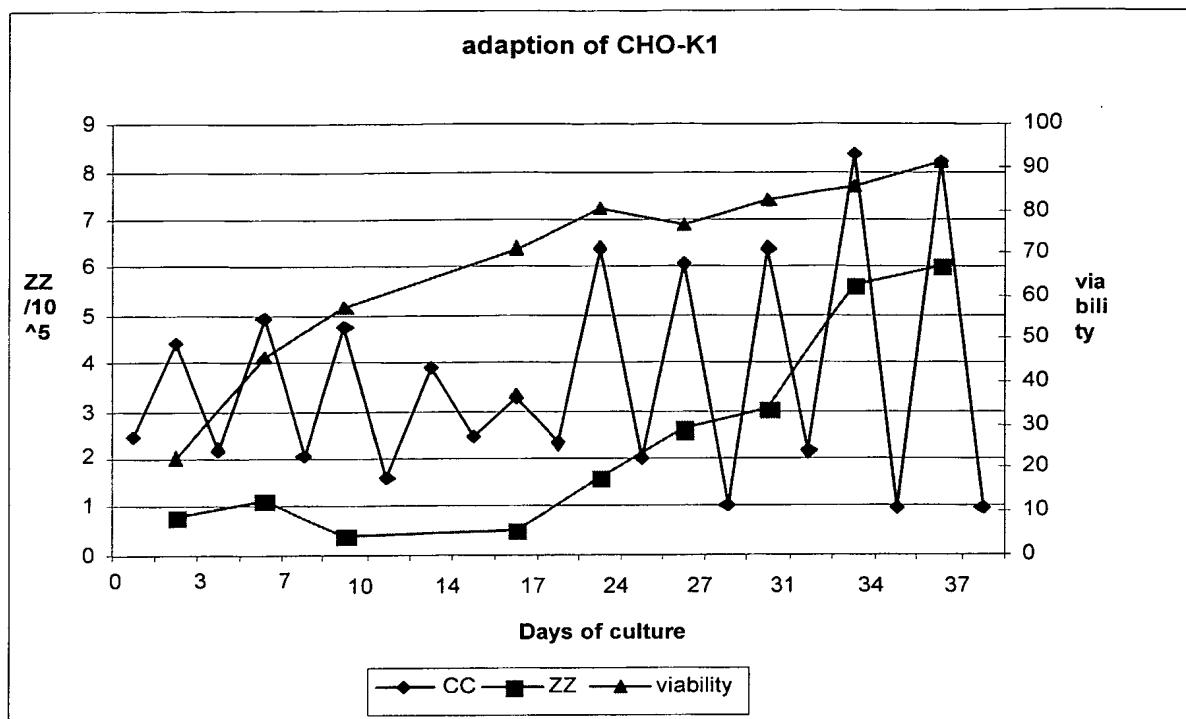


Fig. 1

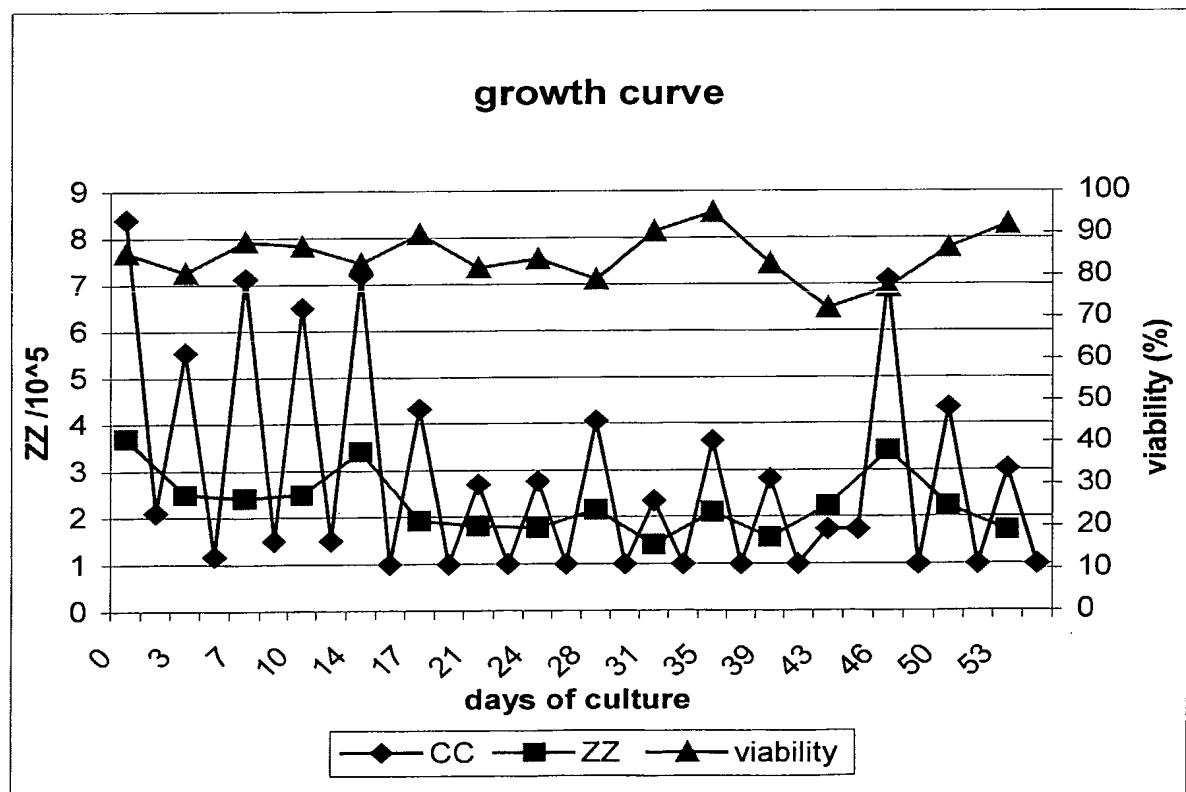
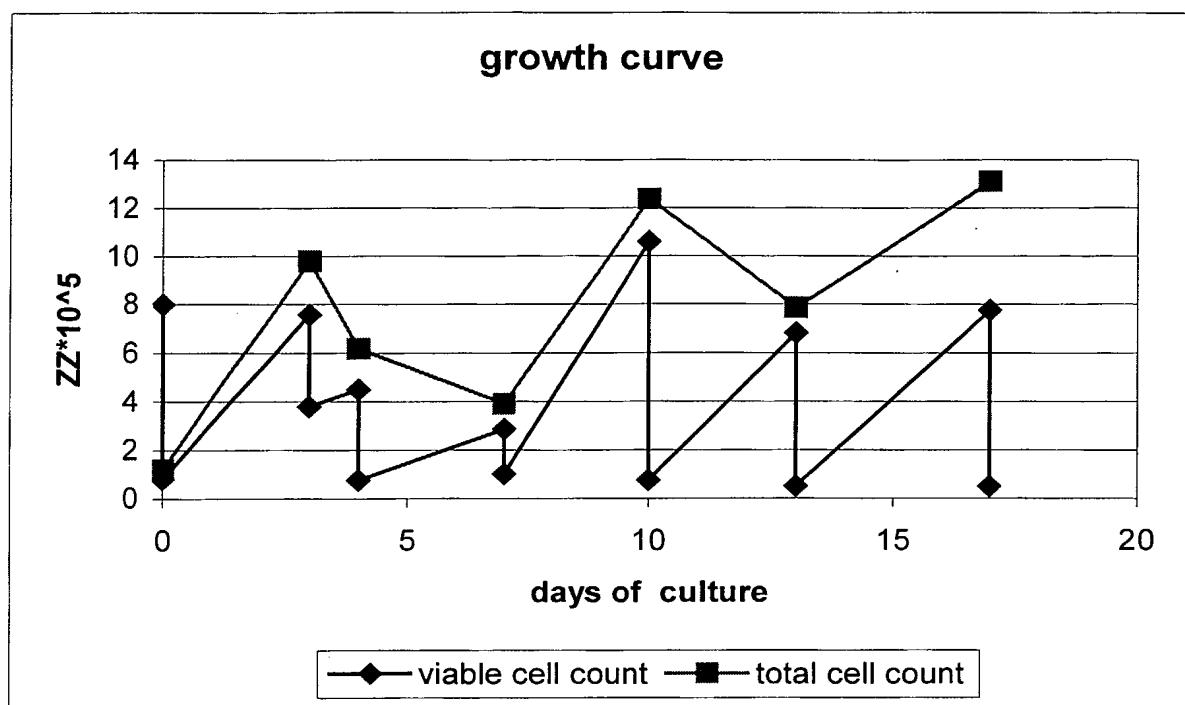
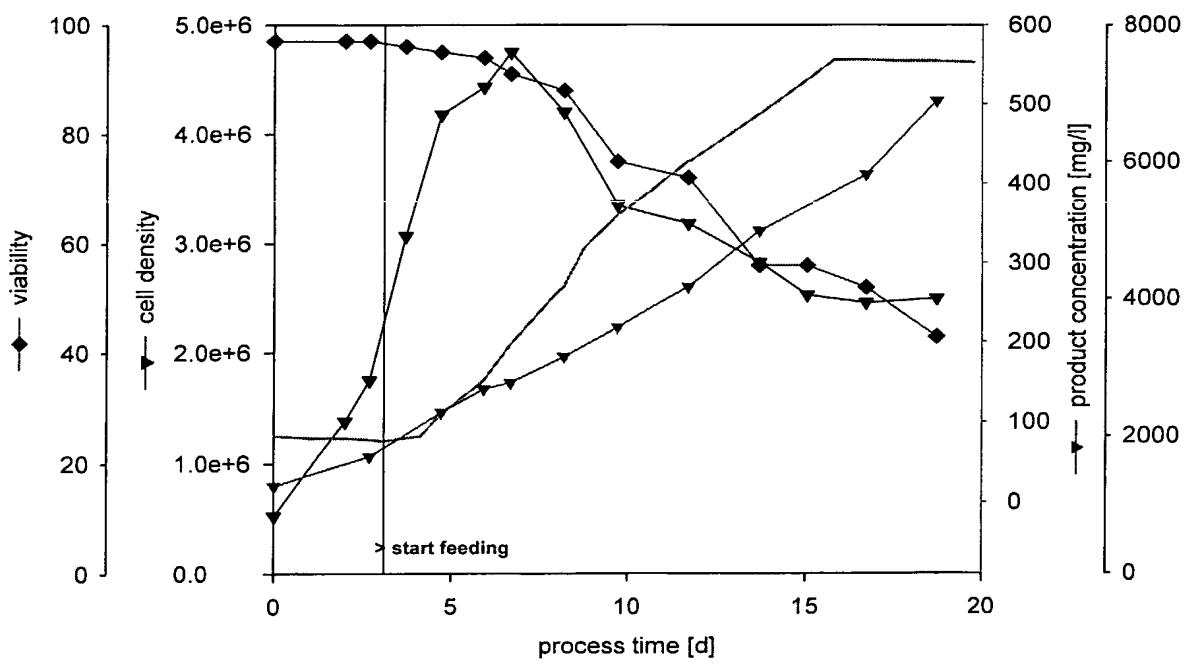


Fig. 2

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**Fig. 3****Fig. 4**

- 3/4 -

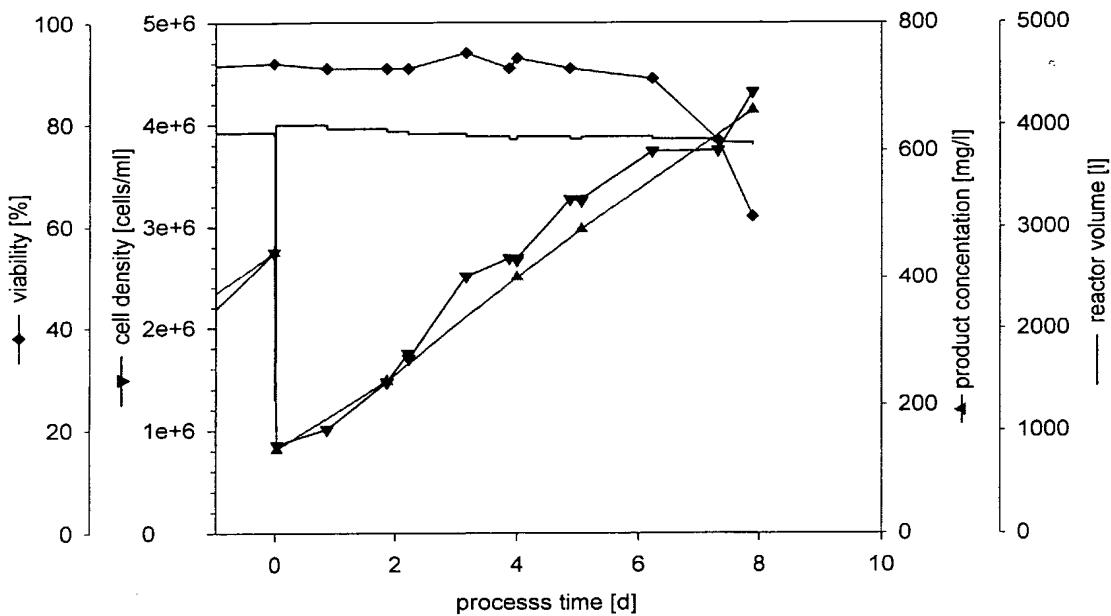


Fig. 5

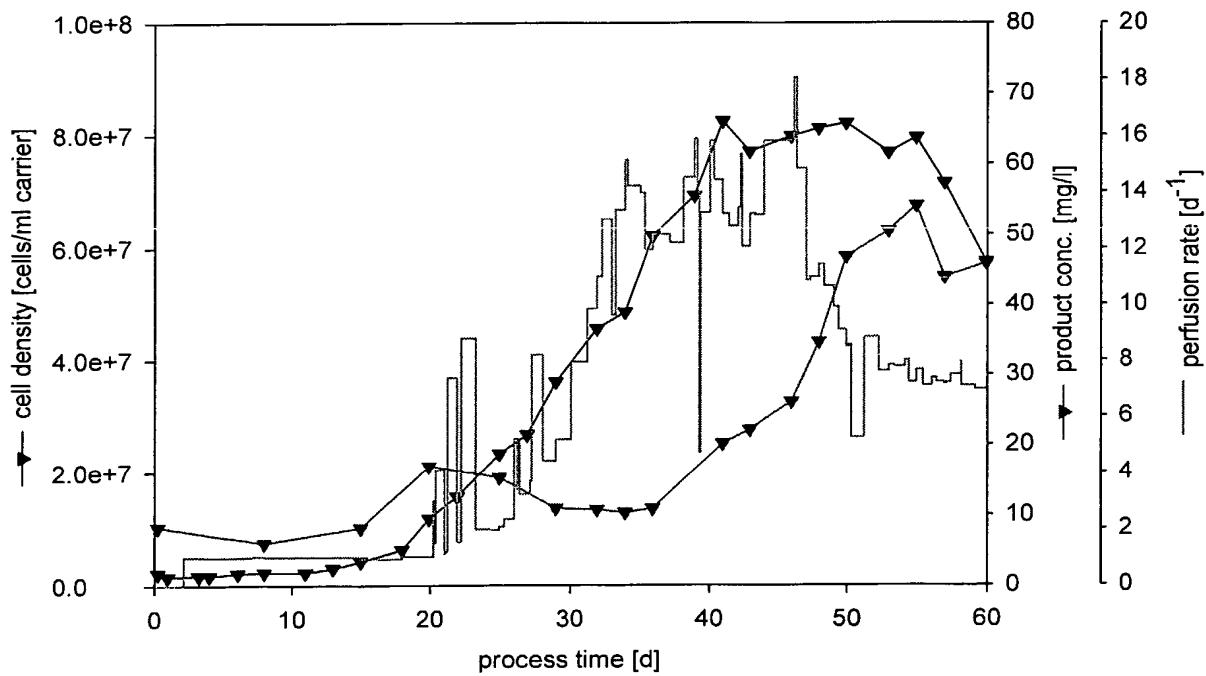


Fig. 6

- 4/4 -

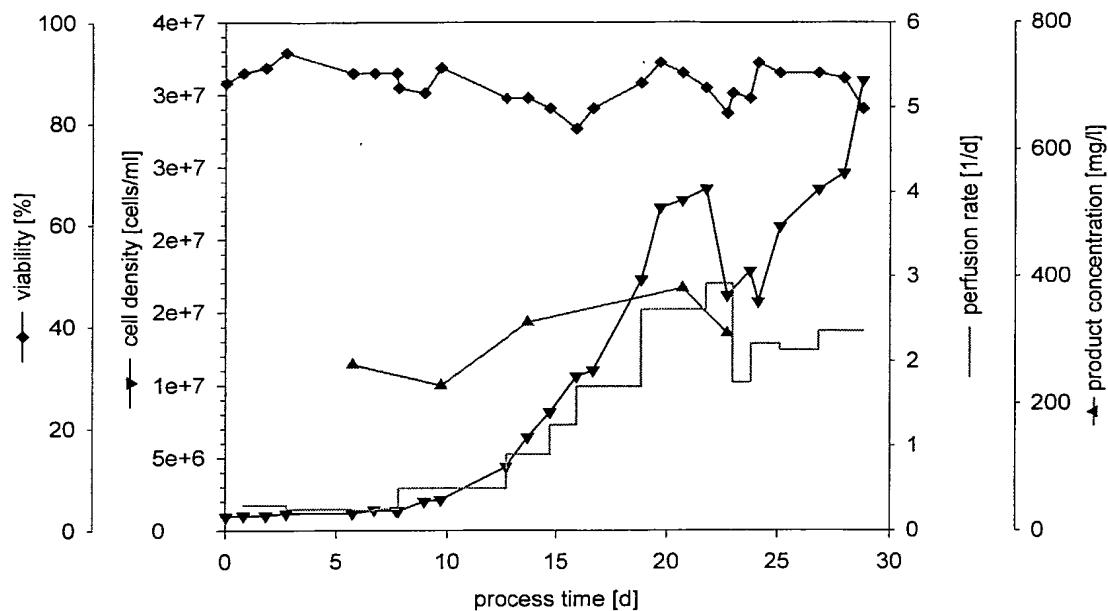


Fig. 7

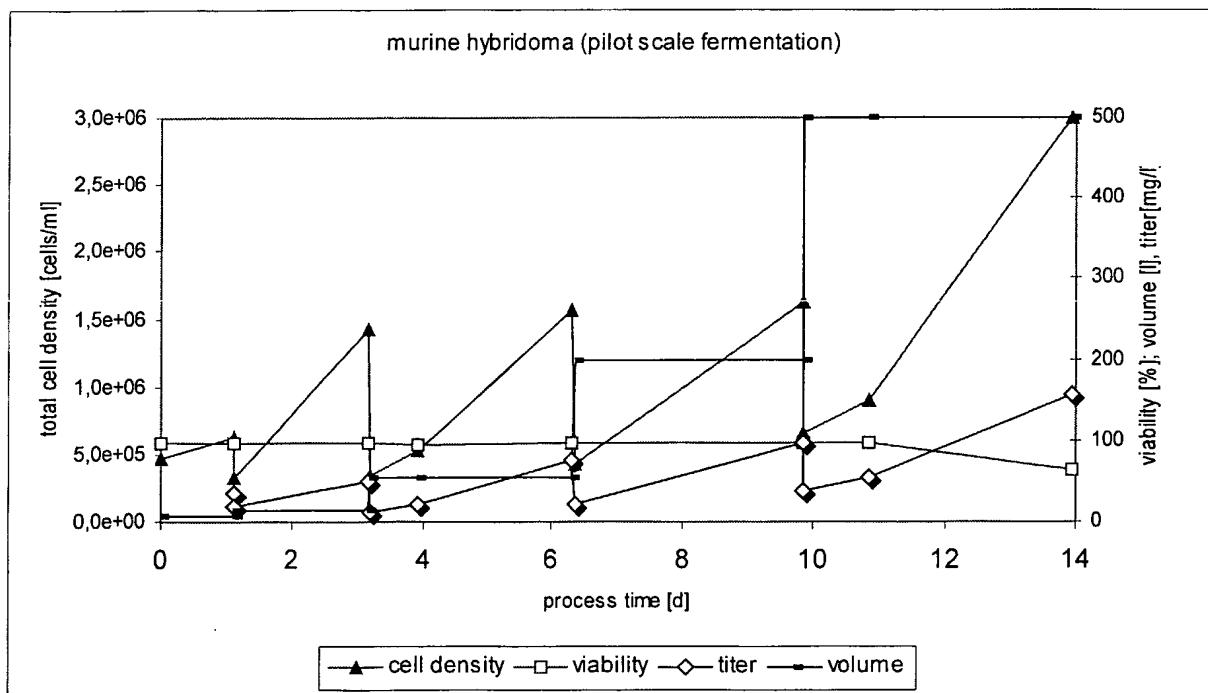


Fig. 8

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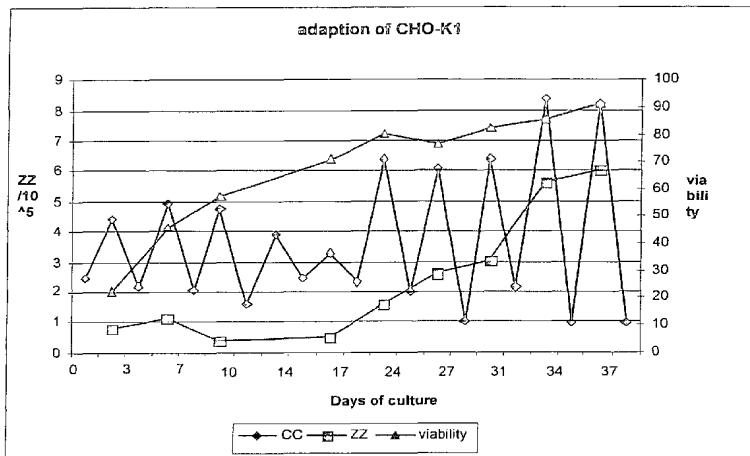
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WO 03/046162 A3

(57) Abstract: The invention provides a method for producing a polypeptide of interest which method comprises: providing a transformed host cell which comprises a nucleotide sequence which encodes a polypeptide of interest and which directs expression of the polypeptide of interest in the transformed host cell, or a hybridoma which comprises a nucleotide sequence encoding a polypeptide of interest; providing a culture medium suitable for mammalian cell culture; and culturing the hybridoma or transformed host cell in the culture medium under conditions that allow for expression of the polypeptide of interest, wherein the culture medium (i) comprises water, a buffer, an energy source, amino acids, a lipid source or precursor, a source of iron, non-ferrous metal ions, inorganic salts, vitamins and cofactors; and (ii) is free from each of a plant-derived or animal-derived peptone, a functional protein, and a full-length polypeptide. The invention further relates to a transformed host cell and to suitable low-cost media applicable in the method of the present invention.



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16 October 2003

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 02/13431

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 7 C12N5/00 C12P21/02 C12P21/08 C12N5/10 C12N5/12
 C07K14/505

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 IPC 7 C12N C12P C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, COMPENDEX, BIOSIS, MEDLINE, EMBASE, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	STOLL T S ET AL: "Systematic improvement of a chemically-defined protein-free medium for hybridoma growth and monoclonal antibody production" JOURNAL OF BIOTECHNOLOGY, ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL, vol. 45, no. 2, 28 February 1996 (1996-02-28), pages 111-123, XP004036827 ISSN: 0168-1656 see in particular Table 1 the whole document ----	1-8, 16, 18-25, 29-31
Y	----- -/-	9-15, 17, 26

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

° Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

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INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZANG M ET AL: "PRODUCTION OF RECOMBINANT PROTEINS IN CHINESE HAMSTER OVARY CELLS USING A PROTEIN-FREE CELL CULTURE MEDIUM" BIO/TECHNOLOGY, NATURE PUBLISHING CO. NEW YORK, US, vol. 13, 1 April 1995 (1995-04-01), pages 389-392, XP002032677 ISSN: 0733-222X the whole document -----	1-15, 18-23, 29-31
Y	US 4 965 199 A (LEVINSON ARTHUR D ET AL) 23 October 1990 (1990-10-23) the whole document -----	9-15, 17, 23-25
Y	SUNG KWAN YOON ET AL: "INFLUENCE OF REDUCING AGENTS ON THE SECRETION RATE OF RECOMBINANT ERYTHROPOIETIN FROM CHO CELLS" BIOTECHNOLOGY LETTERS, KEW, SURREY, GB, vol. 20, no. 2, February 1998 (1998-02), pages 101-104, XP009007462 ISSN: 0141-5492 the whole document -----	9-15, 23-25
X	US 6 048 728 A (INLOW DUANE ET AL) 11 April 2000 (2000-04-11) the whole document -----	19-26

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 02/13431

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.: 27, 28, 32-39 because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-26 (entirely) and 29-31, (partially)

Remark on Protest

- The additional search fees were accompanied by the applicant's protest.
- No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

Invention 1: claims 1-26 (entirely),
29-31 (partially)

Method for producing a polypeptide of interest which method comprises providing a hybridoma or a transformed host cell which comprises a nucleotide sequence which encodes a polypeptide of interest and which directs expression of the polypeptide of interest in the transformed host cell; providing a culture medium suitable for mammalian cell culture; and culturing the hybridoma or transformed host cell in the culture medium under conditions that allow for expression of the polypeptide of interest, characterized in that the culture medium (i) comprises water, a buffer, an energy source, amino acids, a lipid source or precursor, a source of iron, non-ferrous metal ions, inorganic salts, vitamins and cofactors; and (ii) is free from each of a plant-derived or animal-derived peptone, a functional protein, and a full-length polypeptide; corresponding medium and use thereof.

2. Claims: invention 2: claims 27 and 28 (entirely),
29-31 (partially)

Medium suitable for freezing and/or maintaining a mammalian cell, characterized in that it consists of water, a buffer, preferably PBS, and at least one cryoprotective agent, preferably selected from the group consisting of dimethylsulfoxide (DMSO) and polyvinylpyrrolidone (PVP)

3. Claims: invention 3: claims 32-38

A transformed host cell that comprises
(a) a first polynucleotide vector comprising a first nucleotide sequence that encodes a polypeptide of interest, and, optionally, a second nucleotide sequence that encodes a selectable marker; and a second polynucleotide vector comprising a third nucleotide sequence that encodes a different selectable marker and that is free from a nucleotide sequence encoding a polypeptide of interest; or, alternatively,
(b) a polynucleotide vector comprising a first nucleotide sequence that encodes a polypeptide of interest, and, optionally, a second nucleotide sequence that encodes a selectable marker, and further a third nucleotide sequence that encodes a different selectable marker; and, optionally,
(c) at least one more polynucleotide vector comprising a nucleotide sequence encoding another polypeptide of interest and, optionally, a nucleotide sequence encoding a selectable

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

marker;
the polynucleotide vectors being integrated into the genome
of the transformed host cell.

Invention 4: claim 39 (entirely)

A composition comprising a polypeptide of interest obtained
in a method as defined in any one of claims 1-18, the
polypeptide of interest preferably being selected from the
group consisting of human growth hormone, human monoclonal
antibodies, preferably of subclasses IgG, IgM, and IgA, and
monoclonal human/mouse chimeric antibodies.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 27, 28, 32-39

Present claims 1-18 and 29-31 relate to an extremely large number of possible cell lines. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only methods involving CHO cells and murine hybridomas (see examples). In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to CHO cell lines and murine hybridomas. Claim 8 is not supported by the description within the meaning of Article 6 PCT since the claimed ranges are not found in the description.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

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